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BIOCHEMICAL NITROGEN FIXATION STUDIES. III. PRODUCTION AND OXIDATION OF ETHYL ALCOHOL BY LEGUME NODULES[1]

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(WITH ONE FIGURE)

Introduction

It was shown in the first paper of this series (1) that when detached legume nodules are cultured for a short time (either with or without added glucose) under conditions allowing sufficient oxygenation of the tissues, a respiratory quotient approximating 1.00 is obtained. This respiratory quotient (R.Q.) corresponds to the complete oxidation of sugar. If the oxygenation is insufficient, however, the R.Q. is greater than 1.00 and becomes infinite in the limiting case where no molecular oxygen is available, since CO_2 is still given off. Such respiratory quotients correspond to the splitting off of CO_2 from the sugar, leaving as a residue one or more complex compounds. Since it is well known that ethyl alcohol is produced by most plant tissues under such conditions, it seemed desirable to determine to what extent, if any, it is also produced by nodules, and if so whether or not it is oxidized by them when the tissues are fully oxygenated. This paper gives the results obtained, and in addition certain results with legume and non-legume roots which were used for comparison.

Methods

WARBURG MANOMETRIC METHOD.—The Warburg manometric procedure as used in the work with nodules has already been described (1[2]). Its suitability for the

[1] The writers are indebted to Dr. R. T. MILNER and Mrs. MILDRED S. SHERMAN for advice concerning the alcohol determinations, to Mrs. SHERMAN for most of the determinations, and to Dr. DEAN BURK for criticism of certain parts of the text.

[2] In the preceding two papers of this series (pp. 518 and 536), the symbols Q_{O_2}, $\acute{Q}_{O_2}$, $Q_{O_2}(N)$, and R.Q. are defined erroneously in terms of ml. O_2 or CO_2 per mg. per hour, whereas the correct definition should have been expressed in terms of cu. mm. per mg. per hour.

present study depends chiefly on the fact that the R.Q. (0.67) corresponding to complete oxidation of alcohol is quite different from that (1.00) of nodules respiring aerobically without external substrate or with sugar. Therefore if varying quantities of alcohol be added to the nodules and it is oxidized, corresponding variations of the R.Q. can be expected, from a value of 1.00 where no alcohol is added, to 0.67 where enough is added to replace sugar entirely in respiration. The range of variation in R.Q. values obtained for nodules by this technique, as may be noted from the tables that follow, is greater than is usually observed with bacterial cultures, owing partly to greater difficulty in getting uniform samples and partly at least to the higher CO_2 values of the blanks, and the resulting greater gross variation. While too much significance must not be given to the absolute magnitude of some of the results, they are in general clear cut and ample for the conclusions drawn.

CHEMICAL METHOD.—While the manometric method can be expected to give fairly conclusive results as to the oxidation of alcohol under aerobic conditions, the evidence is indirect; in any case it cannot do more than indicate the general nature (average molecular formula) of the residual compounds resulting from the fermentative splitting off of CO_2 from sugar under anaerobic conditions. It seemed advisable, therefore, to determine chemically the accumulation or disappearance of alcohol under both anaerobic and aerobic conditions. For this purpose a micro adaptation of CLARK's modification (7) of the Vieböck and Schwappach method for the determination of methoxy and ethoxy groups was used. In principle the method depends upon the production of a volatile iodide by boiling the material in hydriodic acid. The iodide is distilled off, decomposed, and the resulting iodine determined volumetrically. The method seems to be practically specific for alcohol in plant tissues, because of all the groups (methoxy, ethoxy, isopropoxy, and cyclopropoxy) that will produce volatile iodides under the conditions named, only ethoxy can ordinarily be expected as a volatile compound in such tissues. Trial runs on pure alcohol solution gave recoveries within the errors of the independent information concerning its concentration. A slight failure of complete recovery is of negligible importance in a study such as this, since the controls and the experimental cultures are affected alike and the differences are affected very little, if at all. The alcohol was separated from the tissues and solution by distillation.

Nodules or roots of the following plant species were used: cowpea (*Vigna sinensis* (L.) Endl.), Korean lespedeza (*Lespedeza stipulacea* Maxim.), crotalaria (*Crotalaria spectabilis* Roth.), soybean (*Soja max* (L.) Piper), common white sweet clover (*Melilotus alba* Desr.), a yellow-flowered sweet clover (*M. dentata* (Waldst. and Kit.) Pers.), hairy vetch (*Vicia villosa* Roth.), common vetch (*V. sativa* L.), crown vetch (*Coronilla varia* L.), red clover (*Trifolium pratense* L.), oats

(*Avena sativa* L.), corn (*Zea mays* L.), and narrow-leaved plantain (*Plantago lanceolata* L.).

Results

Anaerobic production of alcohol by nodules

Data on the anaerobic production of alcohol by nodules are given in table 1. The experiments lasted about 2.5–4.7 hours and were conducted in special large vessels which allowed the CO_2 to be determined manometrically on samples large enough for the chemical determination of alcohol. The results are the means of two cultures except where specified otherwise, but the controls consisted of single lots of 10 gm. each of nodules. In calculating the CO_2 produced it was necessary

TABLE 1

ALCOHOL AND CO_2 PRODUCTION BY NODULES UNDER ANAEROBIC CONDITIONS

Exp. no.	Plant	Nodules used (gm.)	Glucose added (%)	Gas used	CO_2 produced (mg.)	C_2H_5OH			Equivalent to CO_2 produced (mg.)
						At start (mg.)	At close (mg.)	Produced (mg.)	
44	Cowpea	5	2.00	N_2	4.27	0.60	4.17	3.57	4.47
45	Soybean	5	1.14	N_2	10.16	1.04	9.88	8.84	10.63
45	Soybean*	5	0.00	N_2	7.02	1.04	7.43	6.39	7.35

* One culture only.

to include that produced after the vessels were filled with nitrogen and before the first readings were taken. This amount was estimated on the assumption that production of CO_2 during the interval before the readings were begun was at the same rate at which it occurred afterward. The last column of the table records the amount of alcohol that would have been produced to correspond to the CO_2 found if hexose were the substrate affected and if only CO_2 and alcohol were produced by its breakdown. The values are included to give an idea of the relative abundance of alcohol and of other products of fermentation produced by the nodules.

The following points seem to be fairly well established by the data in the table: (1) alcohol is probably always present in small amounts in the nodules under usual conditions; (2) alcohol accumulates under anaerobic conditions; and (3) assuming that hexose is the substance broken down to CO_2 and unoxidized compounds, alcohol is by far the most abundant of the latter, averaging in these experiments about 83 per cent of them. These results held whether or not glucose was added in the medium, but in its presence the quantity of alcohol produced may have been increased to some extent.

Aerobic oxidation of alcohol by nodules

The aerobic oxidation of alcohol was investigated by both manometric and direct chemical methods. Table 2 shows the effect on R.Q. and on Q_{O_2} of the addition of different amounts of alcohol to the medium, and table 3 records the effect of glucose and alcohol combined. Figure 1 shows the means of the R.Q.'s for the five experiments with cowpea nodules recorded in table 2. The effect of greater amounts of alcohol than 5 per cent was investigated in only one experiment, because in this instance 10 per cent appeared to be toxic, as evidenced by a considerable reduction of oxygen uptake (Q_{O_2}).

Table 2 and figure 1 show that a progressively smaller decrease in R.Q. resulted from each increase in alcohol concentration, until the R.Q. was reduced almost to that (0.67) characteristic of the complete oxidation of alcohol.

The last two lines in table 2 show the relative value of the Q_{O_2} with various percentages of alcohol, together with the number of experiments on which each value is based. Below the toxic range of the material the Q_{O_2} is increased by a mean maximum of about 5 per cent or 6 per cent, as compared with about 12 per cent for glucose. It does not follow, however, that only 5 per cent or 6 per cent of the oxygen consumed is used in the oxidation of alcohol, for if such were the case the R.Q. would not be reduced below 0.98. That it actually approached 0.67 with little change in Q_{O_2} suggests rather strongly that the alcohol reduced the amount of other substrate oxidized.

The results in table 3 show that the effects of alcohol and of glucose on the R.Q. are exerted in the presence as well as in the absence of the other, the final R.Q. when both are added being intermediate between that resulting from alcohol alone and that from glucose alone. The R.Q. values greater than 1.00 observed in the controls and glucose-only cultures indicate that, in spite of the use of practically pure oxygen as the aerating material, complete aerobiosis was not attained—except possibly with the crotalaria nodules. Indeed, with these nodules the values were abnormally low throughout. The table shows also that both substrates increased the Q_{O_2} in the presence as well as in the absence of the other, the increase when both were added being not much more than the sum of the increases produced by adding each alone. It is probable that this relationship would not hold if each were present in the concentration at which it produces its maximum effect.

These data show that alcohol and glucose are both acceptable substrates for oxidation by the nodules, and that each substance competitively inhibits the oxidation of the other. Furthermore, the fact that very small concentrations of alcohol reduce the R.Q.'s markedly and do not change the Q_{O_2}'s in proportion constitutes strong evidence that it—rather than glucose—is the compound preferentially oxidized.

In order to check by direct methods the oxidizability of alcohol by nodules,

TABLE 2

OXIDATION OF ADDED ALCOHOL BY NODULES: MANOMETRIC RESULTS

Exp. no.	Plant	Gas used		Control		Percentage alcohol in medium* (without glucose)						
				With 1% glucose	Without glucose	0.02	0.1	0.5	1	2	5	10
28.....	Korean lespedeza	Air	R.Q.	1.37	1.17	.....	.....	0.94	.....	0.87	.....	.....
			Q_{O_2}	1.80	1.69	.....	.....	1.77	.....	1.72	.....	.....
30.....	Korean lespedeza	80% O_2 20% N_2	R.Q.	1.03	0.97	.....	.....	.....	.....	0.66	.....	.....
			Q_{O_2}	5.13	4.48	.....	.....	.....	.....	4.70	.....	.....
31.....	Korean lespedeza	Air	R.Q.	.....	1.26	.....	.....	1.05	.....	0.98	.....	.....
			Q_{O_2}	.....	1.50	.....	.....	1.40	.....	1.66	.....	.....
		80% O_2 20% N_2	R.Q.	.....	1.01	0.93	0.86	0.80	.....	0.68	.....	0.50
			Q_{O_2}	.....	4.22	4.46	3.98	4.17	.....	4.06	.....	2.65
36.....	White sweet clover	O_2	R.Q.	0.95	1.03	.....	.....	.....	0.69	.....	.....	.....
			Q_{O_2}	4.84	3.83	.....	.....	.....	4.13	.....	.....	.....
50.....	Yellow sweet clover	O_2	R.Q.	1.09	1.06	1.01	0.94	0.96	0.96	0.86	0.75	.....
			Q_{O_2}	6.61	6.03	6.11	5.97	6.06	5.02	5.77	5.61	.....
49.....	Cowpea	O_2	R.Q.	1.04	1.03	0.98	0.81	0.69	0.64	0.71	0.67	.....
			Q_{O_2}	2.79	2.45	2.21	2.74	3.06	3.03	3.01	2.86	.....
55.....	Cowpea	O_2	R.Q.	1.00	1.03	0.92	0.84	0.81	0.72	0.65	0.65	.....
			Q_{O_2}	3.76	3.91	3.89	3.69	3.94	3.81	3.65	3.76	.....
56.....	Cowpea	O_2	R.Q.	1.11	1.05	1.00	0.84	0.77	0.74	0.73	0.75	.....
			Q_{O_2}	3.88	3.24	3.56	3.55	3.91	3.64	3.65	3.60	.....
57.....	Cowpea	O_2	R.Q.	1.06	1.10	1.04	0.86	0.80	0.71	0.74	0.66	.....
			Q_{O_2}	4.33	3.74	3.98	4.12	4.22	4.35	4.16	3.94	.....
58.....	Cowpea	O_2	R.Q.	0.95	0.98	0.92	0.83	0.77	0.62	0.65	0.75	.....
			Q_{O_2}	5.81	5.47	5.72	5.95	5.80	5.60	6.12	5.82	.....
Mean of experiments with cowpea		O_2	R.Q.	1.03	1.04	0.97	0.84	0.77	0.69	0.70	0.70	.....
			Q_{O_2}	4.11	3.76	3.87	4.01	4.19	4.09	4.12	4.00	.....
Mean of all experiments available.....		No. of experiments		9	....	7	7	9	7	15	6	1
		Relative	Q_{O_2}	112	100	103	103	106	103	106	103	63

* Most of these results are based on one culture only, but most of the no-alcohol and 2 per cent-alcohol figures are the means from two cultures.

some experiments were conducted in which alcohol was added to the culture medium. The amount present in nodules and medium at the end of an incuba-

TABLE 3

OXIDATION OF ADDED ALCOHOL BY NODULES IN PRESENCE OF ADDED GLUCOSE: MANOMETRIC RESULTS

Exp. no.	Plant	Gas used		Substrate additions			
				Glucose 1%	Nothing	Glucose 1%, alcohol 2%	Alcohol 2%
75	Cowpea	O_2	R.Q.	1.28	1.17	0.84	0.81
			QO_2	6.37	5.80	6.77	6.22
78	Crotalaria	O_2	R.Q.	1.04	0.95	0.75	0.56
			QO_2	7.95	6.19	7.84	6.21
86	Hairy vetch	O_2	R.Q.	1.21	1.11	1.04	0.97
			QO_2	8.20	8.16	9.15	9.53
	Mean	O_2	R.Q.	1.18	1.08	0.87	0.78
			QO_2	7.51	6.72	7.92	7.32
			Relative QO_2	112	100	118	109

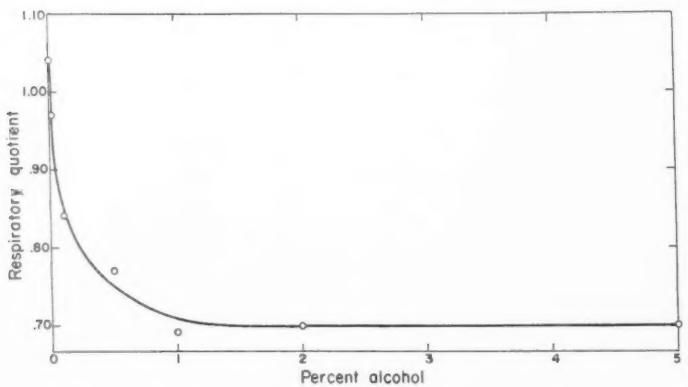

FIG. 1.—Effect of alcohol on respiratory quotient of cowpea nodules

tion period was then compared with that present at the beginning. For this purpose 3-gm. lots of nodules were placed with 5-cc. portions of 0.2 per cent alcohol in culture medium in tightly stoppered 125-cc. Erlenmeyer flasks in an atmosphere

of oxygen at room temperature, 24°–25.5° C., and shaken on a shaking machine during culture periods of 3–7 hours. Controls were distilled with as little delay as possible and without previous exposure to pure O_2. It is believed that no significant error was introduced by the slight unavoidable delay in distilling the last of the controls, since a further control experiment showed little change in the alcohol content of nodules after several hours in air at room temperature. The results are given in table 4, which shows that the data of the chemical analyses agree with those from the manometric work in indicating that alcohol in culture medium is oxidized by legume nodules under suitable conditions.

TABLE 4

OXIDATION OF ADDED ALCOHOL BY NODULES: CHEMICAL RESULTS

EXP. NO.	PLANT	GAS USED	DRY WT. NODULES (GM.)	C_2H_5OH		
				MEAN CONTENT OF CONTROLS (MG.)	MEAN CONTENT OF INCUBATED SAMPLES (MG.)	OXIDIZED PER GM. DRY MATTER PER HOUR (MG.)
60	Cowpea*†	O_2	0.615	7.81	4.73‡	1.00
60	Cowpea†§	O_2	0.615	7.09	4.66‡	0.79
61	Cowpea*‖	O_2	0.534	7.21	1.91‡	1.99
61	Cowpea§¶	O_2	0.534	6.50	2.93‡	1.34

* Nodules crushed.
† Two controls and two experimental cultures.
‡ Calculated to 5 hours' duration.
§ Nodules not crushed.
‖ One control and one experimental culture.
¶ Three controls and three experimental cultures.

ALCOHOL PRODUCTION AND OXIDATION BY PLANT ROOTS

It seemed reasonable to conclude from the work with nodules that they behave toward alcohol fundamentally like ordinary root tissues, since it is generally accepted that most plant tissues produce alcohol anaerobically. It was thought advisable, however, to check the point definitely by testing root tissues of various species. Small roots of both legumes and non-legumes were investigated for both alcohol production and alcohol consumption.

The roots were treated essentially in the same manner as the nodules, except that in the Erlenmeyer flask technique for detection of alcohol consumption the roots were occasionally shaken by hand instead of constantly by machine, since when machine shaking was attempted the roots of the culture aggregated into a thick clump in the middle of the vessel instead of remaining well distributed throughout the liquid. The duration of the experiments varied from 2.5 to 5.5 hours.

The results for alcohol production are given in table 5. It is clear from the data that, as already shown for nodules, ethyl alcohol and CO_2 are produced in the small roots of both legumes and non-legumes under anaerobic conditions, and that the proportions of the two are roughly those to be expected if the main reaction concerned is a fermentative splitting of sugar into the two compounds. As a mean for the three kinds of roots used, the alcohol produced was about 85 per cent of the amount equivalent to the CO_2 produced.

The results for the aerobic utilization of alcohol by roots secured by the manometric method are given in table 6 and those by the analytical method for experiments of about 3–4.5 hours' duration in table 7. They show that roots also

TABLE 5

ALCOHOL AND CO_2 PRODUCTION BY ROOTS UNDER ANAEROBIC CONDITIONS

EXP. NO.	PLANT	ROOTS USED (GM.)	GLUCOSE ADDED (%)	GAS USED	CO_2 PRODUCED (MG.)	C_2H_5OH			
						AT START (MG.)	AT CLOSE (MG.)	PRODUCED (MG.)	EQUIVALENT TO CO_2 PRODUCED (MG.)
44	Cowpea*	5	2.00	N_2	5.92	1.30	6.25	4.95	6.20
45	Soybean*	5	1.14	N_2	7.07	0.76	6.98	6.22	7.40
47	Corn†	4	1.00	N_2	3.61	0.31	3.80	3.49	3.78

* One culture and one (10 gm.) control.

† Two cultures and two (8 gm.) controls.

oxidized alcohol aerobically. Thus legume and non-legume roots behaved alike in this respect. They show further that the Q_{O_2} was increased by alcohol in both legume and non-legume roots, but that—as with nodules—the increase was not great enough to account for the change in R.Q. It is likely that the alcohol reduced the amount of other substrate oxidized. One point of difference between legume nodules and roots, previously mentioned (2), is that the roots consistently gave a lower R.Q. than the nodules, the R.Q. for roots being less than 1.00 under aerobic conditions. This may mean (1) that the food reserves in the small roots are normally so low that they are quickly exhausted, with consequent oxidation of nitrogenous compounds; (2) that they include considerable amounts of some compound (possibly alcohol) other than sugar and which on complete oxidation has an R.Q. considerably less than unity; or (3) that incomplete oxidation of sugar occurs, with the formation of compounds with R.Q. values greater than 1.00. As will appear later, the evidence seems to favor the second of these possibilities.

TABLE 6

OXIDATION OF ADDED ALCOHOL BY ROOTS: MANOMETRIC RESULTS

EXP. NO.	PLANT	GAS USED		CONTROL		PERCENTAGE ALCOHOL IN MEDIUM* (WITHOUT GLUCOSE)						
				WITH 1% GLUCOSE	WITHOUT GLUCOSE	0.02	0.1	0.5	1	2	5	10
32	Korean lespedeza	Air	R.Q.	0.97	0.80	.....	.....	.....	.....	0.73	.....	.....
			QO_2	2.35	1.50	.....	.....	.....	.....	1.93	.....	.....
		80% O_2, 20% N_2	R.Q.	0.94	0.68	.....	.....	.....	.....	0.58	.....	.....
			QO_2	3.01	2.06	.....	.....	.....	.....	2.09	.....	.....
33	Korean lespedeza	Air	R.Q.	.....	0.85	.....	.....	.....	.....	0.65	.....	.....
			QO_2	.....	1.64	.....	.....	.....	.....	1.59	.....	.....
		80% O_2, 20% N_2	R.Q.	.....	0.79	0.73	0.69	0.65	0.66	0.69	.....	0.69
			QO_2	.....	1.60	1.89	1.85	1.89	1.67	1.92	.....	0.59
53	Oat	O_2	R.Q.	0.96	0.92	0.87	0.86	0.81	0.80	0.79	0.66	.....
			QO_2	2.05	1.75	1.73	1.70	1.62	1.68	1.53	1.16	.....
54	Oat	O_2	R.Q.	0.90	0.92	0.81	0.82	.....	.....	0.75	0.80	.....
			QO_2	2.19	1.94	2.07	2.01	.....	.....	2.06	1.99	.....
Mean of experiments with oat		O_2	R.Q.	0.93	0.92	0.84	0.84	.....	.....	0.77	0.73	.....
			QO_2	2.12	1.85	1.90	1.86	.....	.....	1.80	1.58	.....
Mean with Korean lespedeza		Relative	QO_2	151	100	118	116	118	104	111	.....	037
Mean with oat		Relative	QO_2	115	100	103	101	93	96	97	85	.....
Mean with both species		Relative	QO_2	132	100	108	105	105	100	106	85	.....

* Most of the results are from one culture only, but most of the no-alcohol and 2 per cent-alcohol figures are the means from two cultures.

TABLE 7

OXIDATION OF ADDED ALCOHOL BY ROOTS: CHEMICAL RESULTS

EXP. NO.	PLANT	GAS USED	DRY WT. ROOTS (GM.)	C_2H_5OH*		
				MEAN CONTENT OF CONTROLS (MG.)	MEAN CONTENT OF INCUBATED SAMPLES (MG.)	OXIDIZED PER GM. DRY MATTER PER HOUR (MG.)
62	Cowpea	O_2	0.394	13.10	12.55†	0.28
63	Red clover	O_2	0.410	13.83	12.23†	0.78
64	Narrow-leaved plantain	O_2	0.453	6.84	4.63†	0.98

* Means of four controls and four experimental cultures.

† Calculated to 5 hours' duration.

Effect of oxygen on consumption of respirable carbohydrate

If it be admitted, as the data already given strongly indicate, that the chief compounds concerned in fermentation and respiration of plant tissues are carbohydrate and alcohol, it is possible to calculate from the CO_2 and O_2 data certain functions which throw light on the processes concerned. In tables 8 and 9 certain such functions[3] are listed to show the effect of varying the oxygen concentration on the respiration of nodules and roots with different amounts of added glucose and alcohol.

It will be noted that with all the tissues used and with or without added alcohol or glucose, both CO_2 production and O_2 consumption were increased by increased

[3] The formulas for the calculation of these functions depend for fermentation and oxidation on the following equations:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \text{ (fermentation of carbohydrate)}$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2 \text{ (oxidation of carbohydrate)}$$

$$2C_2H_5OH + 6O_2 \rightarrow 6H_2O + 4CO_2 \text{ (oxidation of alcohol)}$$

The formulas are derived as follows: Following a common convention, a unit of carbohydrate is the amount which will produce a unit (in this instance 1 cu. mm. at standard temperature and pressure) of CO_2 by fermentation. It is also the amount which will produce three units (3 cu. mm.) of CO_2 by oxidation, since—as shown by the first two equations—on a molal or volume basis three times as much CO_2 is produced by the oxidation of carbohydrate as by its fermentation. In oxidation of carbohydrate, the CO_2 evolved and O_2 consumed are equal and therefore interchangeable in calculation.

A unit of alcohol is the quantity produced by the fermentation of a unit of carbohydrate; therefore alcohol accumulated (per hour) equals $Q_{CO_2} - Q_{O_2}$. This follows because it equals the total CO_2 produced minus the CO_2 of oxidation, and the CO_2 of oxidation equals the O_2 consumed. When carbohydrate is the substrate the value for alcohol accumulated is positive and is the same as that for carbohydrate fermented but not metabolized further; when alcohol is the substrate the value of course is negative.

Similarly carbohydrate consumed equals $Q_{CO_2} - \frac{2}{3}Q_{O_2}$, for where carbohydrate is the substrate it equals the carbohydrate fermented to alcohol ($Q_{CO_2} - Q_{O_2}$) plus that oxidized ($\frac{1}{3}Q_{O_2}$); and where alcohol is present also and being oxidized the formula remains the same since the CO_2 produced is two-thirds of the O_2 consumed.

Ratio of CO_2 evolved under any condition of aeration to CO_2 evolved anaerobically is evidently $\frac{Q_{CO_2}}{Q^{N_2}_{CO_2}}$. This is the reciprocal of the $\frac{I}{N}$ or $\frac{NR}{OR}$ ratio sometimes mentioned in work on plant respiration. The ratio as used here, however, seems to serve the purpose of the present study better than the more common form.

Decrease (due to oxygen) in alcohol accumulated equals $Q^{N_2}_{CO_2} - Q_{CO_2} + Q_{O_2}$; that is, it equals alcohol accumulated (or carbohydrate fermented) anaerobically ($Q^{N_2}_{CO_2}$) minus that ($Q_{CO_2} - Q_{O_2}$) accumulated at the oxygen concentration concerned.

Decrease (per unit of substrate oxidized) in alcohol accumulated equals $\frac{Q^{N_2}_{CO_2} - Q_{CO_2} + Q_{O_2}}{\frac{2}{3}Q_{O_2}}$, for it obviously equals the decrease, due to oxygen, in alcohol accumulated ($Q^{N_2}_{CO_2} - Q_{CO_2} + Q_{O_2}$) divided by substrate oxidized ($\frac{1}{3}Q_{O_2}$). The substrate oxidized may be either carbohydrate, alcohol, or a mixture of the two.

It should be mentioned that, by the use of these terms and functions, nothing is implied concerning the nature of the first step in normal respiration, or, in general, the mechanism or path of the process.

TABLE 8

EFFECT OF OXYGEN CONCENTRATION ON RESPIRATION OF LEGUME NODULES

1	2	3	4	5	6	7	8	9	10	11	12
Exp. no.	Plant	Substrate added	Gas used	CO_2 evolved	O_2 consumed	Respiratory quotient	Alcohol accumulated	Carbohydrate consumed	Ratio of CO_2 evolved to CO_2 evolved anaerobically	Decrease due to oxygen, in alcohol accumulated	Decrease per unit of substrate oxidized in alcohol accumulated
				Q_{CO_2}	Q_{O_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	$Q_{CO_2}-Q_{O_2}$	$Q_{CO_2}-\frac{1}{3}Q_{O_2}$	$\frac{Q_{CO_2}}{Q^{N_2}_{CO_2}}$	$Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}$	$\frac{Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}}{\frac{1}{4}Q_{O_2}}$
25.....	Soybean	2% glucose	N_2	0.77	.....	.....	0.77	0.77	1.00	0.00	.....
			7.4% O_2, 92.6% N_2	1.41	0.93	1.52	0.48	0.79	1.82	0.29	0.94
			Air	1.84	1.49	1.23	0.35	0.85	2.38	0.42	0.85
			50% O_2, 50% N_2	2.34	2.34	1.00	0.00	0.76	3.02	0.77	0.99
			O_2	3.35	3.16	1.06	0.19	1.24	4.33	0.58	0.55
26.....	Soybean	2% glucose	N_2	0.76	.....	.....	0.76	0.76	1.00	0.00	.....
			50% O_2, 50% N_2	2.60	2.17	1.20	0.43	1.15	3.42	0.33	0.46
			O_2	3.24	2.94	1.10	0.30	1.26	4.26	0.46	0.47
27.....	Common vetch	2% glucose	N_2	0.90	.....	.....	0.90	0.90	1.00	0.00	.....
			7.4% O_2, 92.6% N_2	2.20	1.69	1.30	0.51	1.07	2.44	0.39	0.69
			Air	3.40	3.45	0.98	−0.05	1.10	3.77	0.95	0.83
			50% O_2, 50% N_2	5.78	5.73	1.01	0.05	1.96	6.40	0.85	0.45
			80% O_2, 20% N_2	7.82	7.60	1.03	0.22	2.75	8.66	0.68	0.27
			O_2	8.01	7.82	1.02	0.19	2.80	8.87	0.71	0.27
27.....	Common vetch	Nothing	Air	2.78	2.80	1.00	−0.02	0.91	.....	.....	.....
			80% O_2, 20% N_2	6.13	6.31	0.97	−0.18	1.92	.....	.....	.....
16.....	Crown vetch	Nothing	Air	2.77	2.41	1.15	0.36	1.16	.....	.....	.....
			O_2	5.47	5.06	1.08	0.41	2.10	.....	.....	.....

TABLE 8—*Continued*

1	2	3	4	5	6	7	8	9	10	11	12
Exp. no.	Plant	Substrate added	Gas used	CO_2 evolved	O_2 consumed	Respiratory quotient	Alcohol accumulated	Carbohydrate consumed	Ratio of CO_2 evolved to CO_2 evolved anaerobically	Decrease due to oxygen, in alcohol accumulated	Decrease per unit of substrate oxidized in alcohol accumulated
				Q_{CO_2}	Q_{O_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	$Q_{CO_2}-Q_{O_2}$	$Q_{CO_2}-\frac{3}{5}Q_{O_2}$	$\frac{Q_{CO_2}}{Q^{N_2}_{CO_2}}$	$Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}$	$\frac{Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}}{\frac{1}{2}Q_{O_2}}$
30.....	Korean lespedeza	Nothing	Air	1.75	1.58	1.11	0.17	0.70	.....	.....	.....
			80% O_2, 20% N_2	4.35	4.48	0.97	−0.13	1.36	.....	.....	.....
			O_2	4.74	5.01	0.94	−0.27	1.40	.....	.....	.....
30.....	Korean lespedeza	1% glucose	Air	2.41	1.72	1.40	0.69	1.26	.....	.....	.....
			80% O_2, 20% N_2	5.28	5.13	1.03	0.15	1.86	.....	.....	.....
			O_2	5.51	5.45	1.01	0.06	1.88	.....	.....	.....
30.....	Korean lespedeza	5% glucose	Air	3.32	0.43	7.68	2.89	3.03	.....	.....	.....
			80% O_2, 20% N_2	4.68	4.36	1.07	0.32	1.77	.....	.....	.....
			O_2	5.38	5.04	1.07	0.34	2.02	.....	.....	.....
31.....	Korean lespedeza	Nothing	Air	1.88	1.50	1.26	0.38	0.88	.....	.....	.....
			80% O_2, 20% N_2	4.20	4.16	1.01	0.04	1.43	.....	.....	.....
31.....	Korean lespedeza	0.5% alcohol	Air	1.48	1.40	1.05	0.08	0.55	.....	.....	.....
			80% O_2, 20% N_2	3.33	4.17	0.80	−0.84	0.55	.....	.....	.....
31.....	Korean lespedeza	2.0% alcohol	Air	1.63	1.66	0.98	−0.03	0.52	.....	.....	.....
			80% O_2, 20% N_2	2.77	4.06	0.68	−1.29	0.06	.....	.....	.....

TABLE 9

EFFECT OF OXYGEN CONCENTRATION ON RESPIRATION OF LEGUME AND OF NON-LEGUME ROOTS

1	2	3	4	5	6	7	8	9	10	11	12
Exp. no.	Plant	Substrate added	Gas used	CO_2 evolved	O_2 consumed	Respiratory quotient	Alcohol accumulated	Carbohydrate consumed	Ratio of CO_2 evolved to CO_2 evolved anaerobically	Decrease due to oxygen, in alcohol accumulated	Decrease per unit of substrate oxidized in alcohol accumulated
				Q_{CO_2}	Q_{O_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	$Q_{CO_2}-Q_{O_2}$	$Q_{CO_2}-\frac{1}{3}Q_{O_2}$	$\frac{Q_{CO_2}}{Q^{N_2}_{CO_2}}$	$Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}$	$\frac{Q^{N_2}_{CO_2}-Q_{CO_2}+Q_{O_2}}{\frac{1}{3}Q_{O_2}}$
32.....	Korean lespedeza	Nothing	N_2	0.48	.....	.....	0.48	0.48	1.00	0.00	.....
			Air	1.20	1.50	0.80	−0.30	0.20	2.52	0.78	1.56
			80% O_2, 20% N_2	1.40	2.06	0.68	−0.66	0.03	2.94	1.14	1.66
32.....	Korean lespedeza	1% glucose	N_2	0.81	.....	.....	0.81	0.81	1.00	0.00	.....
			Air	2.29	2.35	0.97	−0.06	0.72	2.83	0.87	1.11
			80% O_2, 20% N_2	2.81	3.01	0.94	−0.20	0.80	3.47	1.01	1.01
32.....	Korean lespedeza	2% alcohol	N_2	0.60	.....	.....	0.60	0.60	1.00	0.00	.....
			Air	1.41	1.93	0.73	−0.52	0.12	2.33	1.12	1.74
			80% O_2, 20% N_2	1.20	2.09	0.58	−0.89	−0.19	1.99	1.49	2.14
33.....	Korean lespedeza	Nothing	Air	1.39	1.64	0.85	−0.25	0.30	.....	.....	.....
			80% O_2, 20% N_2	1.27	1.60	0.79	−0.33	0.20	.....	.....	.....
33.....	Korean lespedeza	0.5% alcohol	Air	1.16	1.74	0.66	−0.58	0.00	.....	.....	.....
			80% O_2, 20% N_2	1.22	1.89	0.65	−0.67	−0.04	.....	.....	.....
33.....	Korean lespedeza	2.0% alcohol	Air	1.04	1.59	0.65	−0.55	−0.02	.....	.....	.....
			80% O_2, 20% N_2	1.33	1.92	0.69	−0.59	0.05	.....	.....	.....

concentrations of oxygen. This general result could be produced either by the complete oxidation of a greater proportion of the substrate consumed, by the consumption of more substrate, or by both. The R.Q.'s given in the two tables indicate that the result was at least partly due—up to a certain point—to more complete oxidation and after that to increased consumption of substrate, for in the case of nodules supplied with glucose the R.Q.'s decreased with increased oxygen concentration until a value of approximately 1.00 was reached, and then remained constant. With roots the data are less complete and less conclusive, but indicate a similar behavior, with a somewhat lower minimum value; in particular, where alcohol was added this minimum appears to have been about 0.67.

The fact that CO_2 evolution and O_2 consumption increased in the upper range of O_2 concentrations, while the R.Q. remained constant or decreased, shows that in this range substrate consumption was increased by increases in concentration of oxygen. The question as to whether or not additional O_2 increased consumption of substrate in the low range of oxygen concentrations, where more complete oxidation is also a factor, is answered for nodules with added glucose in columns 8 and 9 of table 8. It is shown here that, although alcohol accumulation decreased with increased O_2 concentration, carbohydrate consumption increased throughout the entire range of oxygen concentrations. For nodules with added alcohol, however, and for roots—both with and without added alcohol (table 9)—the situation with regard to carbohydrate consumption was reversed; that is, less carbohydrate was consumed at high oxygen concentrations than at low ones, although the alcohol accumulation continued to decline. In fact the alcohol accumulation values became negative, indicating a consumption of alcohol. While the data are hardly sufficient to be positive, it seems very likely that here also the increase in O_2 concentration led to an increase in total substrate consumed, but with alcohol as the main substrate.

The observation just given concerning the effect of increased oxygen concentration on the consumption of carbohydrate is verified by the figures in column 10 of both tables, where the amount of CO_2 evolved at different oxygen concentrations is compared with that evolved anaerobically in those experiments which included anaerobic cultures. With either nodules or roots with glucose added, in all concentrations of oxygen producing complete aerobiosis (where the R.Q. equaled approximately 1.00) the ratio of CO_2 evolved aerobically to that evolved anaerobically was 3.00 or greater. This means that if the substrate being utilized was glucose, as is usually considered to be the case in plant respiration, as much or more glucose was consumed in the presence of oxygen as in its absence, since only three times as much CO_2 is produced by the complete oxidation of carbohydrate as by its fermentation to alcohol. The size of this quotient is of less significance where alcohol was added, of course, since in that case the original substrate appears to have been chiefly this compound.

This relationship can be analyzed further by the functions given in columns 11 and 12. Column 11 gives the decrease, due to oxygen, in alcohol accumulated, and column 12 gives the amount of this decrease per unit of substrate oxidized. This latter function, sometimes known as the Meyerhof oxidation quotient, equals 1.00 if the effect of the oxygen is merely to oxidize alcohol (or similar intermediate); equals less than 1.00 if in addition carbohydrate consumption is increased; and equals more than 1.00 if carbohydrate consumption is decreased or alcohol disappears more rapidly than it is oxidized to CO_2 and H_2O (as, for example, by a coupled oxidative resynthesis to carbohydrate such as has been observed occasionally).

In all cases, either with nodules or roots, where glucose was added the decrease in alcohol accumulated was 1.00 or less for each unit of substrate oxidized. The evidence therefore indicates that under these circumstances oxygen stimulated the consumption of glucose. On the other hand, in the one instance with roots respiring only their own food reserves and in the one where alcohol was added, the decrease in alcohol accumulated per unit of substrate oxidized was definitely greater than 1.00. From a consideration of the respiratory quotients, mentioned earlier, it seems likely that the result is due to a decrease in carbohydrate consumption rather than to a resynthesis of carbohydrate from alcohol. Unfortunately no data are at hand which would permit the calculation of this function for nodules cultured in alcohol, but there is no reason to suspect that they would be contradictory.

Some of these conclusions are naturally only tentative, because of the limited amount of work on which they are based.

Discussion

Similarity between nodules and small roots in formation and utilization of alcohol

The well-known difference in the nitrogen metabolism of roots and of nodules with their inclosed bacteria shows that some sort of fundamental difference exists between the nitrogen-fixing tissue and the ordinary root tissue. The results reported here, in agreement with the data given in the second paper (**2**) of this series, indicate strongly that so far as respiration is concerned no such differences exist. It is true that the respiratory quotient is lower in roots and that a sparing action of oxygen on carbohydrate is evident, as opposed to the opposite effect in nodules, but the experiments indicate that the only thing necessary to bring about the difference is a difference in the amounts of carbohydrate and alcohol (intermediate) present in the tissues. The differences appear to be those of degree only, and not of kind, with the respiration process fundamentally the same in the two tissues. The roots respire like nodules supplied with alcohol, although in view of data already presented (**2**) the question is still open as to whether the results are

due to an abundance of alcohol or to a lack of carbohydrate, with accompanying oxidation of protein. The oxidation of protein, however, would imply that the roots were oxidizing themselves away, a thing that seems improbable in growing organs. On the other hand, the oxidation of alcohol or other intermediate would raise the question as to where it comes from, since it is generally understood that carbonaceous foods are transported from the green tissues as sugar. But when it is considered that only fine roots were used, this question loses much of its importance, since the substrate had to traverse the thick roots in which, as shown by one or two experiments not otherwise mentioned in this paper, the conditions were at least partially anaerobic. Consequently it does not seem unreasonable to suppose that some of the substrate may have undergone fermentation during its passage, so that much of that which reached the fine roots was in the form of alcohol.

Role of alcohol in respiration

It has long been recognized that in the absence of oxygen, alcohol and CO_2 are the chief products of the respiration of sugars by most higher plant tissues, and that in its presence the corresponding products are CO_2 and water. The related point—as to whether or not alcohol is an intermediate product in normal aerobic respiration—has often been discussed but cannot yet be said to be settled. In connection with this point, the oxidizability of alcohol in the tissues is of fundamental importance, for obviously the compound can scarcely function as an intermediate if it cannot be oxidized. Early work on this phase of the question was done by Mazé and Perrier (**14**), Kostytschew (**9, 10**), and Zaleski (**21**), all of whom gave evidence that alcohol is oxidizable in the tissues of higher plants. Takahashi (**16**), however, had previously implied by analogy with animal tissues (but without experimental proof) that it is more slowly oxidized than sugar, and Kostytschew's results included some cases in which no oxidation occurred. He concluded that alcohol is oxidized slowly and incompletely; and his later attitude (**11**) seems to have been dominated even more by this idea, since he said that he "has shown that ethyl alcohol is oxidized by seed plants with difficulty or not at all." Moreover, the same idea seems to prevail in much of the contemporary work on the subject (**3, 11, 15, 17**).

In the last decade, however, scattered data have appeared which indicate that the early results were correct. Bugajewsky (**4**) showed that alcohol accumulates in germinating pea seeds in the absence of oxygen and disappears in the presence of the latter, and Chatterji (**6**) likewise showed that the CO_2 output of certain leaves is increased by infiltrating them with dilute solutions of alcohol. While these results do not prove that oxidation of alcohol took place in the plant tissues, they do point that way. Wetzel (**20**) and Thunberg (**18**) have investigated the enzyme system involved. Thunberg showed that the seeds of many plants con-

tain an alcohol dehydrogenase, and WETZEL claimed to have found in some plants a complete enzyme system for the complete oxidation of alcohol through acetaldehyde and acetic acid. (Unfortunately the experimental basis for the claim is not given in the only paper (**20**) discussing it that the writers have seen.)

The results obtained in the present study definitely favor the idea that alcohol is oxidizable in the tissues of higher plants. It was found to accumulate in the tissues under anaerobic conditions and to disappear under aerobic conditions, and the R.Q. of tissues supplied alcohol artificially was such as would be expected if the alcohol which disappeared had been oxidized to CO_2 and H_2O. Moreover, the ratio of aerobically produced to anaerobically produced CO_2, and the decrease —caused by oxygen—in alcohol accumulated per unit of carbohydrate oxidized when an abundance of carbohydrate was present, indicate that no more alcohol disappeared under the influence of oxygen than was oxidized to H_2O and CO_2. The most reasonable explanation for the disappearance of the alcohol therefore seems to be not that it was resynthesized to a fermentable substrate but that it was oxidized to CO_2 and water. The evidence therefore indicates that, whether or not alcohol normally occurs as an intermediate compound in the aerobic respiration of carbohydrate, it can be oxidized completely by many higher plant tissues.

The results also indicate that oxygen may exert a sparing effect or no sparing effect on the carbohydrate respired in one and the same tissue, apparently depending on the relative abundance of carbohydrate and intermediate present in the tissue. Since the R.Q.'s suggest that the sparing effect when present was not due to any oxidative reconversion of intermediates, the suggestion remains that in tissues in which much intermediate has accumulated the O_2 in some way reduces the primary breakdown of the carbohydrate. This concept is supported by the fact that alcohol and sugar appear to be competitively oxidized in these tissues, with a probable preferential oxidation of alcohol.

It is not entirely certain that the sparing effect[4] discussed here is identical with the one sometimes reported in plant tissues, but there is apparently no special reason for thinking that it is not; and if it should be found to be the same it would explain in many cases, without assuming any fundamental difference in the mechanism of respiration, why the sparing effect sometimes occurs and sometimes does not. In that case it will be found if for some reason intermediates of fermentative origin have accumulated sufficiently and will be absent if intermediates are absent, or are present in small amounts only. MARSH and GODDARD (**12**) have recently reported in carrot tissue a sparing effect of oxygen, accompanied by

[4] Some of the phenomena under discussion here bear resemblance to the frequently discussed Pasteur reaction. However, a considerable amount of uncertainty seems to exist concerning the exact definition of the Pasteur reaction and of some of the terms commonly used in discussing it (**5, 8, 19**). For this reason it has seemed best to discuss the significance of the data without reference to this reaction as such and so far as possible with descriptive terms instead of with the less definite conventional ones.

R.Q. values at 5 per cent O_2 or above, of usually less than 0.90. Apparently the sparing effect in this species also may be dependent upon the accumulation of intermediates in the tissue.

Summary

A series of experiments by both manometric and analytical methods has been conducted on the production and oxidation of ethyl alcohol by legume nodules and the data compared with similar data from small roots of legumes and of non-legumes. The following results were obtained:

1. Ethyl alcohol was present in small amounts in all tissues studied. Under anaerobic conditions it accumulated and CO_2 was evolved in approximately the proportions which would result from an alcoholic fermentation of sugar.

2. Under aerobic conditions, with alcohol added to the medium, part of the alcohol disappeared in a few hours and the R.Q. of the tissues was reduced by an amount depending on the concentration of the alcohol. At the higher concentrations it was commonly reduced nearly to that R.Q. (0.67) corresponding to the complete oxidation of alcohol. These results indicate that the alcohol which disappeared was oxidized completely.

3. Added alcohol increased oxygen consumption of both nodules and roots but not nearly enough to account for the change in R.Q. It is suggested that the alcohol consumption replaced and reduced the consumption of the other substrate present.

4. When glucose and alcohol were made available together in the medium, each affected the R.Q. in the same way as it did in the absence of the other, again without affecting the oxygen consumption proportionately, thus indicating that each inhibited competitively the oxidation of the other.

5. For nodules, either without added substrate or with glucose added, and for small roots with glucose added, the absence of a sparing action of oxygen on carbohydrate (and consequently of any oxidative reconversion of the products of fermentation to carbohydrate or similar compound) was indicated (*a*) by increased CO_2 evolution and O_2 consumption due to increasing concentrations of oxygen; (*b*) by similarly induced increases in carbohydrate consumption; (*c*) by a ratio equal to or greater than 3.00 between the CO_2 produced under complete aerobiosis and that produced anaerobically; and (*d*) by the failure of the decreases, due to oxygen, in alcohol accumulation to exceed the carbohydrate oxidized.

6. On the contrary, for nodules with sufficient added alcohol and for small roots without added substrate or with alcohol added, the presence of a sparing action of oxygen on carbohydrate (in spite of increased total substrate consumption) was indicated by a decrease in carbohydrate consumption with increasing oxygen concentration and by a decrease in alcohol accumulation which was greater than the

carbohydrate oxidized. Since the R.Q.'s indicate the absence of any oxidative reconversion of alcohol, it is suggested that the oxygen may have reduced in some way the primary breakdown of the carbohydrate.

7. The differences between the behavior of nodules and of roots in respiration are not thought to be fundamental. The roots behaved much like nodules with added alcohol.

8. The definite demonstration that alcohol is oxidized by the tissues of higher plants removes an important objection to considering it as an intermediate in normal plant respiration, but of course does not prove that it is an intermediate.

9. It is suggested that the occasionally observed sparing action of oxygen on carbohydrate in respiring plant tissues may be dependent on a high concentration of intermediate. If so, no fundamental difference in the mechanism of respiration need be assumed between that in tissues where the sparing action occurs and that in those from which it is absent.

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ANATOMICAL AND PHYSIOLOGICAL RESPONSES OF THE TOMATO TO VARYING CONCENTRATIONS OF SODIUM CHLORIDE SODIUM SULPHATE, AND NUTRIENT SOLUTIONS

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(WITH TEN FIGURES)

Introduction

Plants grown in soil solutions containing high concentrations of soluble salts commonly exhibit markedly unfavorable growth responses. These may be caused by (*a*) the toxic effect of high concentration of a single ion, (*b*) the interaction of two or more ions present in high concentrations, or (*c*) the combined effect of high total salt concentration.

Experiments were set up to determine (*a*) the character of the anatomical and physiological responses of tomato plants when grown in a series of nutrient solutions containing progressively higher concentrations of the major constituent salts, and (*b*) the responses these plants exhibit when grown in sodium chloride and sodium sulphate solutions having osmotic concentrations equal to those of the solutions in the nutrient series.

Material and methods

The tomato plant was selected because it can be easily grown under greenhouse conditions and has been used extensively in studies of nutrition. The plants were grown in sand cultures in pots devised by EATON (2). These are provided with a reservoir holding approximately 13 liters of solution, which can be circulated at regular intervals by means of compressed air passing through an ejector. A gage on the reservoir indicates the level of the solution, and water was added daily to compensate for losses resulting from transpiration and evaporation. A time clock controls flushing of the cultures, and was set to circulate the liquid at 2-hour intervals from 6:00 A.M. to 6:00 P.M., with one irrigation at midnight. A flow for 3–5 minutes more than displaces the solution held in the sand.

NUTRIENT AND SALT SOLUTIONS

EXPERIMENT I.—A four-salt nutrient solution was used containing $Ca(NO_3)_2$, KNO_3, $MgSO_4$, and KH_2PO_4, and the total salt concentration was adjusted to 0.5 atmosphere osmotic concentration (table 1). This solution was used as the base

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nutrient in the controls and as the initial solution for all cultures until the seeds had germinated. It also constituted the nutrient portion of the sodium chloride and sodium sulphate solutions.

Three series of solutions were used:

1. Base nutrient series: Four solutions in which the nutrient salts were increased at each level to produce osmotic concentrations of 0.5, 1.5, 3, and 4.5 atmospheres.
2. Sodium chloride series: Three solutions consisting of the base nutrient at 0.5 atmosphere plus NaCl sufficient to result in osmotic concentrations of 1.5, 3, and 4.5 atmospheres.
3. Sodium sulphate series: Three solutions consisting of the base nutrient at 0.5 atmosphere plus Na_2SO_4 sufficient to result in osmotic concentrations of 1.5, 3, and 4.5 atmospheres.

TABLE 1

COMPOSITION OF SOLUTIONS USED IN EXPERIMENT I

Concentration of solutions (atmospheres)	Base nutrient series consisting of following salts (millimoles per liter)				Concentration of solutions (atmospheres)	Millimoles added to base nutrient solution at 0.5 atmosphere concentration	
	$Ca(NO_3)_2$	KNO_3	$MgSO_4$	KH_2PO_4		Sodium chloride series NaCl	Sodium sulphate series Na_2SO_4
0.5	2.6	5.2	2.6	0.65	1.5	20.0	15.0
1.5	7.2	14.4	7.2	1.8	3.0	52.0	40.0
3.0	15.8	31.6	15.8	3.95	4.5	89.0	71.0
4.5	24.8	49.6	24.8	6.2			

The proportions of the constituent salts used in the various solutions are expressed in millimoles per liter. In determining the amounts of salts needed to produce 0.5 atmosphere osmotic concentration, c.p. salts and distilled water were used. When commercial salts and Riverside tap water were used, the proportions produce an osmotic concentration of approximately 0.6 atmosphere. Minor elements were added in the following amounts: boron 1.0 p.p.m., zinc 0.05 p.p.m., manganese 0.5 p.p.m., molybdenum 0.05 p.p.m., and copper 0.02 p.p.m. Iron as citrate was supplied as needed in 1 p.p.m. additions.

EXPERIMENT II.—The solutions used were essentially like those in experiment I, but the quantities of salts needed to produce 0.5 atmosphere osmotic concentration were determined on the basis of commercial salts and tap water. The amount of KH_2PO_4 was kept at a constant level (1.8 mm./l.) in all solutions, to avoid the heavy precipitation that occurred in the higher base nutrient cultures in experiment I. In order to explore further the effect of high salt concentrations, an additional culture with salts adjusted to 6 atmospheres osmotic concentration was

added to each series. The base nutrient series was set up at five levels: 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration; and the sodium chloride and sodium sulphate series at four levels: 1.5, 3, 4.5, and 6 atmospheres. The composition of the solutions is indicated in table 2. Minor elements and iron were added as in experiment I, and additional increments of manganese (0.5 and 1 p.p.m.) were supplied during the latter part of the run, when symptoms of manganese deficiency appeared in the leaves of the larger plants.

TABLE 2

COMPOSITION OF SOLUTIONS USED IN EXPERIMENT II

Concentration of solutions (atmospheres)	Base nutrient series consisting of following salts (millimoles per liter)				Concentration of solutions (atmospheres)	Millimoles added to base nutrient solution at 0.5 atmosphere concentration	
	$Ca(NO_3)_2$	KNO_3	$MgSO_4$	KH_2PO_4		Sodium chloride series $NaCl$	Sodium sulphate series Na_2SO_4
0.5	1.3	2.6	1.3	1.8	1.5	20.0	15.0
1.5	7.3	14.6	7.3	1.8	3.0	54.0	40.0
3.0	16.1	32.2	16.1	1.8	4.5	90.0	71.0
4.5	26.0	52.0	26.0	1.8	6.0	120.0	100.0
6.0	35.1	70.2	35.1	1.8			

Replacement and maintenance of solutions

To avoid seedling injury, at the initiation of each experiment all pots were supplied with the base nutrient at the 0.5 atmosphere level, and the higher osmotic concentrations were obtained by a series of increases extending over a 10-day period. Complete changes of solutions were made as follows: experiment I—October 5 and 23, and November 7; experiment II—November 21, December 7 and 26, and January 7. At intervals between solution changes the osmotic concentration of each solution was determined by the freezing-point depression method.

The H-ion concentration was determined with a Beckman pH meter on alternate days, or daily when the plants were larger. Solutions were adjusted to pH 7.0 by additions of HNO_3 or NaOH when there was a variation of more than 0.5 pH. In experiment II, KOH was used instead of NaOH. Until the plants were large, it was never necessary to add more than 5 cc. of HNO_3, and 10 cc. was the greatest amount supplied at one time.

Environmental factors (experiments I and II)

The cultures were grown under greenhouse conditions, and records of temperature and relative humidity were obtained with a hygrothermograph. During the

course of experiment I (September 23 to November 11, 1939) the mean maximum temperature was 88.6° and the mean minimum 61.6° F. Up to the time of the first harvest in experiment II (November 15, 1939 to January 2, 1940) the mean maximum and minimum temperatures were only slightly less than those of experiment I, 86.5° and 59.4° F., respectively. The final 4 weeks of the second experiment (January 2 to 30, 1940) were cooler, 78° and 58.1° F. The mean values for relative humidity were: experiment I—maximum 70.9 per cent, minimum 28.7 per cent; experiment II—77.8 per cent and 39.6 per cent up to the first harvest and 81.3 per cent and 56.1 per cent for the final 4 weeks.

The major climatic variation between the two experiments was the amount of solar and sky radiation. At Riverside, California, the mean total daily radiation for the period of the first experiment was 354.8 gm. calories per square centimeter of horizontal surface, while for the equivalent period of the second experiment it was 256 gm. calories. There was a further reduction in radiation during the final 4 weeks of the second experiment when the daily mean was 221 gm. calories.[3] These values were taken outdoors, and somewhat lower ones would obtain in the greenhouse, where the experiments were conducted.

Planting data (experiments I and II)

A commercial strain of the Marglobe variety of the tomato was used, and all trials were run in duplicate.

The seeds of experiment I were planted September 23, 1939, and the cultures given identical amounts of water and nutrient solution until September 30. At this time all except the control pots were increased to 1.5 atmosphere osmotic concentration. On October 3, all but the controls and 1.5 atmosphere cultures were adjusted to 3 atmosphere concentration; and on October 5, the 4.5 atmosphere cultures were brought to full strength. In this manner the three series were established as follows: the base nutrient series at 0.5 ,1.5, 3, and 4.5 atmospheres osmotic concentration and the sodium chloride and sodium sulphate series at 1.5, 3, and 4.5 atmospheres.

The seeds of experiment II were planted November 15, and on November 25 all except the control pots were adjusted to 1.5 atmosphere concentration. Increases in the concentration of the cultures were made as in experiment I. The 3 atmosphere level was reached November 27, the 4.5 on November 29, and the 6 on December 1. The final adjustment established the three series at the following levels: the base nutrient at 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration and the sodium chloride and sodium sulphate series at 1.5, 3, 4.5, and 6 atmospheres.

[3] These figures supplied through the courtesy of Dr. E. R. Parker, at Citrus Experiment Station, Riverside, where a pyroheliometer is maintained.

Experimentation

Seedling responses

Approximately twenty seedlings were started in each culture. These were thinned selectively to provide three uniform plants. Although the seedlings removed were the least uniform in each culture, their average heights and total fresh and dry weights did provide an index of the early responses to the nutrient and salt solutions used in the three series.

TABLE 3

Experiment II: Seedling harvest data. Fresh and dry weights and percentage of dry matter

Series and concentration of solutions (atmospheres)		Harvest December 7, 1939			Harvest December 13, 1939		
		Fresh wt. 12 plants (gm.)	Dry weight (gm.)	Dry matter (per cent)	Fresh wt. 6 plants (gm.)	Dry weight (gm.)	Dry matter (per cent)
Base nutrient	0.5	11.53	0.84	7.3	22.95	1.81	7.9
	1.5	13.66	1.11	8.1	27.00	1.99	7.4
	3.0	9.05	0.76	8.5	19.31	1.60	8.3
	4.5	5.25	0.47	9.0	6.61	0.64	9.7
	6.0	5.14	0.51	10.0	6.72	0.72	10.8
NaCl	1.5	9.84	0.70	7.2	17.53	1.33	7.6
	3.0	7.05	0.45	6.4	13.24	0.68	5.2
	4.5	7.85	0.55	7.1	12.24	0.72	5.9
	6.0	6.75	0.41	6.1	8.37	0.63	7.6
Na_2SO_4	1.5	8.43	0.67	8.0	20.28	1.54	7.6
	3.0	7.72	0.60	7.9	12.17	0.77	6.4
	4.5	5.80	0.46	8.0	6.24	0.54	8.8
	6.0	3.59	0.31	8.9	3.15	0.28	9.1

In both experiments there was a progressive growth depression with increasing concentration in the sodium chloride and sodium sulphate series. In the base nutrient series the seedlings grown in the 1.5 atmosphere solution were larger and heavier than those in the 0.5 atmosphere culture. In both seedling harvests of experiment II the lower percentages of dry weight in the cultures of the sodium chloride series, as compared with those of equivalent osmotic concentrations in the other two series, indicated a greater degree of succulence in the high chloride solutions (table 3).

Physiological and chemical data

Experiment I.—Three plants in each culture were maintained until November 13, a growing period of 51 days.

Except for the 1.5 atmosphere culture in the base nutrient series, in which the plants were taller than those at the 0.5 level, there was a decrease in height at high

concentrations of the culture solution in all series. The difference in height between the plants in the 1.5 and 4.5 atmosphere solutions was least in the base nutrient and greatest in the sodium sulphate series.

In all series there was a significant reduction in the diameter of the stem at the high concentrations. This was least in the sodium chloride and greatest in the sodium sulphate series (table 4).

Except for the 1.5 atmosphere culture in the base nutrient series, the total fresh and dry weights of the tops decreased at high concentrations. The fact that the 1.5 atmosphere base nutrient solution promoted greater vegetative growth than that of the 0.5 atmosphere culture suggests that the supply of nutrient salts, especially nitrogen, was more nearly optimum at the 1.5 atmosphere level. All cultures,

TABLE 4

EXPERIMENTS I AND II: AVERAGE DIAMETER OF FIRST INTERNODE OF STEM*

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	SERIES					
	BASE NUTRIENT (MM.)		SODIUM CHLORIDE (MM.)		SODIUM SULPHATE (MM.)	
	I	II	I	II	I	II
0.5	10.3	8.0	.....	.....	.....	.....
1.5	9.9	8.2	9.7	8.2	9.0	7.5
3.0	8.8	7.5	9.1	6.8	7.2	6.0
4.5	7.2	6.7	7.5	6.5	4.5	3.2
6.0	.....	6.0	.....	5.4	.....	2.0

* Average of four stems in each culture.

however, except the high concentrations in the base nutrient series, were supplied with the base nutrient solution at the 0.5 atmosphere level, and no symptoms of nitrogen deficiency were observed.

EXPERIMENT II.—Three plants per culture were maintained until January 2, when two plants from each pot were harvested. The length of the growing period, 48 days, was approximately that of experiment I. The remaining plant in each culture was harvested January 30, 75 days after planting. In all series, at the high levels of concentration the stems were shorter and smaller in diameter than at the control or 1.5 atmosphere levels (table 4).

As in experiment I, the fresh and dry weights of tops decreased with increasing concentration, except in the 1.5 atmosphere base nutrient solution (fig. 1).

In both experiments the percentage of dry matter increased with increasing concentration in the sodium sulphate and base nutrient series and remained practically unchanged in the sodium chloride series. This indicates greater succulence in the high chloride plants than in those of the base nutrient and sodium sulphate series at osmotic concentrations (fig. 2).

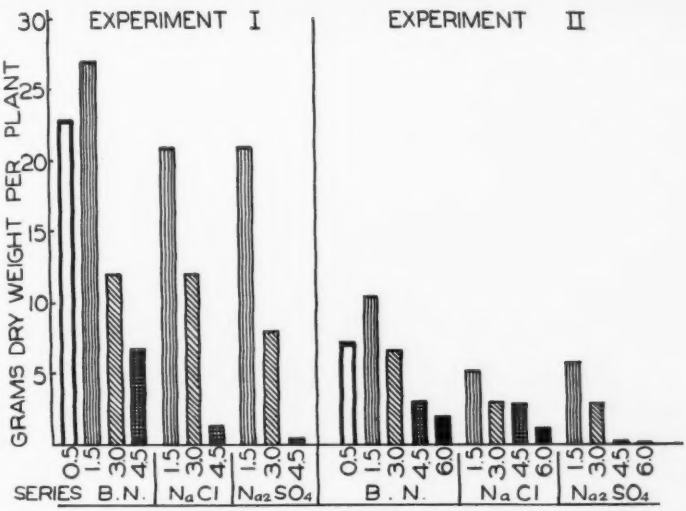
EXPERIMENT I
EXPERIMENT II
GRAMS DRY WEIGHT PER PLANT
30
25
20
15
10
5
0.5 1.5 3.0 4.5 1.5 3.0 4.5 1.5 3.0 4.5 0.5 1.5 3.0 4.5 6.0 1.5 3.0 4.5 6.0 1.5 3.0 4.5 6.0
SERIES B.N. NaCl Na2SO4 B.N. NaCl Na2SO4

FIG. 1.—Experiments I and II. Average dry weights of tops per plant at harvests of November 10 and January 2. Figures below columns indicate atmospheres osmotic concentration of culture solutions.

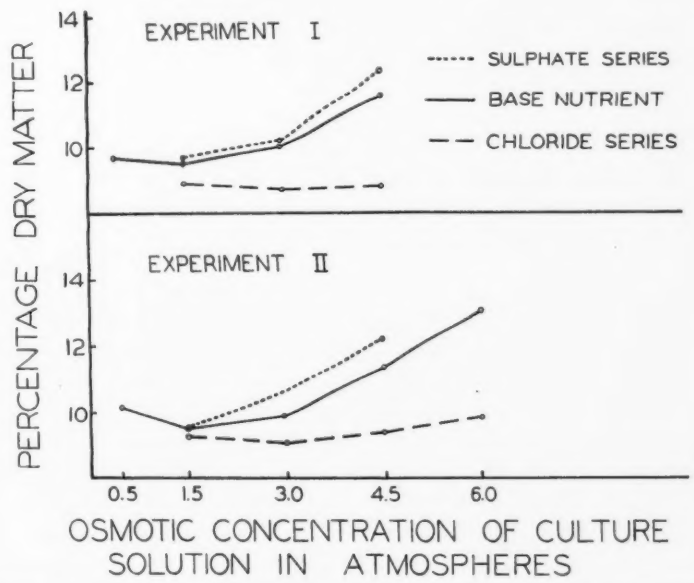

FIG. 2.—Experiments I and II. Percentage dry matter of tops at increasing levels of osmotic concentration in each series.

In corresponding cultures the plants in experiment I were shorter and stockier than those in experiment II and produced greater fresh and dry weights of tops. There was relatively little variation in temperature during the comparable periods of the two experiments, and these differences can probably be ascribed to the light factor, since the solar and sky radiation was 31.5 per cent greater in the first experiment than in the second.

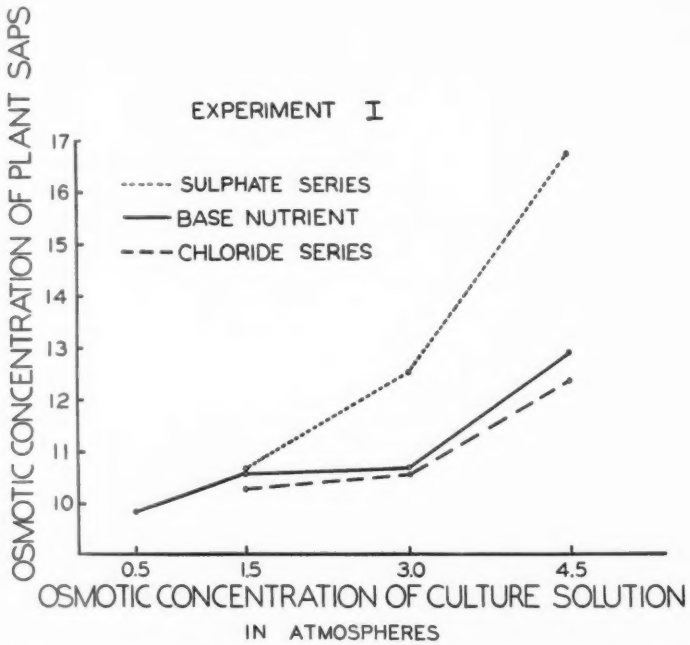

FIG. 3.—Experiment I. Relation between osmotic concentration of plant saps and that of culture solutions.

In both experiments observations on flower-bud formation indicated a definite reduction or retardation in formation in the high sodium chloride solutions and a more pronounced effect in the high sodium sulphate cultures, in which no flower buds were produced up to the time of harvest.

In experiment II all plants except those grown in the 4.5 and 6 atmosphere sodium sulphate solutions had produced evident flower buds at the time of final harvest, but bud formation was retarded at the higher levels in the base nutrient and sodium chloride series. The plants in the 6 atmosphere sodium sulphate solution died before the final harvest. Anthesis was correspondingly delayed at the higher concentrations. The number of inflorescences was greatest in the 1.5 and 3

atmosphere cultures in the base nutrient series and at the 1.5 atmosphere level in the sodium chloride and sodium sulphate series.

The osmotic concentrations of the expressed sap were determined at the conclusion of experiment I. In all series the sap concentration increased with increasing concentration of the culture solution. At isosmotic solution pressures the sap concentrations were highest in the sodium sulphate series. There was close correspondence between the base nutrient and sodium chloride series, the latter having slightly lower sap concentrations (fig. 3).

In experiment II the accumulation of Cl, S, and Na was determined for both harvests of the sodium chloride and sodium sulphate series.[4] Determinations were also made for the 0.5 and 1.5 atmosphere levels of the base nutrient series for purposes of comparison (table 5).

TABLE 5

EXPERIMENT II: BASE NUTRIENT SERIES
ACCUMULATION OF CL, S, AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS					
	FIRST HARVEST			SECOND HARVEST		
	Cl	S as SO_4	Na	Cl	S as SO_4	Na
0.5	197	558	147	154	450	127
1.5	112	634	65	151	681	54

In the sodium chloride series there was increasing accumulation of Na and Cl in the tops of the plants with increasing concentration of the culture solution (table 6). In the sodium sulphate series the accumulation of Na increased progressively at the higher concentrations, but there was no marked trend with respect to S intake (table 7).

The results obtained for S accumulation in the sodium sulphate series are not in agreement with unpublished results obtained for tomatoes by EATON[5] at this laboratory. In out-of-doors sand cultures, he found 150, 177, 222, and 299 m.e. of SO_4 per liter in the sap of tomatoes grown in solutions containing 2.68, 50, 160, and 250 m.e. of SO_4 per liter, respectively.

In both sodium series the intake of Na on a dry-weight basis indicates a significant relation to the amount available in the culture solutions. In solutions with

[4] A.O.A.C. methods for Cl and S analyses.
Na analysis: BARBER, H. H., and KOLTHOFF, I. M., A specific reagent for the rapid gravimetric determination of sodium. Jour. Amer. Chem. Soc. **50**:1625–1631. 1928.

[5] F. M. EATON, private communication.

equal concentrations of Na, the accumulation of Na was greater in the sodium sulphate cultures than in those of the corresponding sodium chloride series (fig. 4). A composite picture of the accumulation of Cl, S, and Na on the basis of dry weight of tops is shown in figure 5.

TABLE 6

EXPERIMENT II: SODIUM CHLORIDE SERIES
ACCUMULATION OF CL AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS			
	FIRST HARVEST		FINAL HARVEST	
	Cl	Na	Cl	Na
1.5	880	653	754	563
3.0	1090	1320	911	934
4.5	1480	1780	1340	1445
6.0	1800	2285	1520	1645

TABLE 7

EXPERIMENT II: SODIUM SULPHATE SERIES
ACCUMULATION OF S AND NA

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MILLIEQUIVALENTS PER KILO DRY WEIGHT OF TOPS			
	FIRST HARVEST		FINAL HARVEST	
	S as SO_4	Na	S as SO_4	Na
1.5	828	1090	694	709
3.0	749	2180	506	1585
4.5	1080	2565	574	2275

ANATOMICAL RESPONSES

At harvest all plants were sampled to obtain representative material for anatomical analysis. In selecting material, care was exercised to establish a definite reference point, so that the structures examined would be comparable. The stem segments were selected from the median portion of the basal internode immediately above the cotyledonary node. Mature leaves were selected, and a rectangular segment of the terminal leaflet, oriented so as to include a portion of the midvein, was taken at a median point in the lamina.

The material was fixed with Navashin's solution, dehydrated in an ethyl-tertiary butyl alcohol series, and infiltrated with a paraffin-beeswax-rubber mix-

ture. Immediately after placing the material in the fixative, air was evacuated from the tissues with a vacuum pump. Sections were cut at 15 μ and stained with a modified Flemming's triple stain. The photomicrographs of all series were taken at the same magnification.

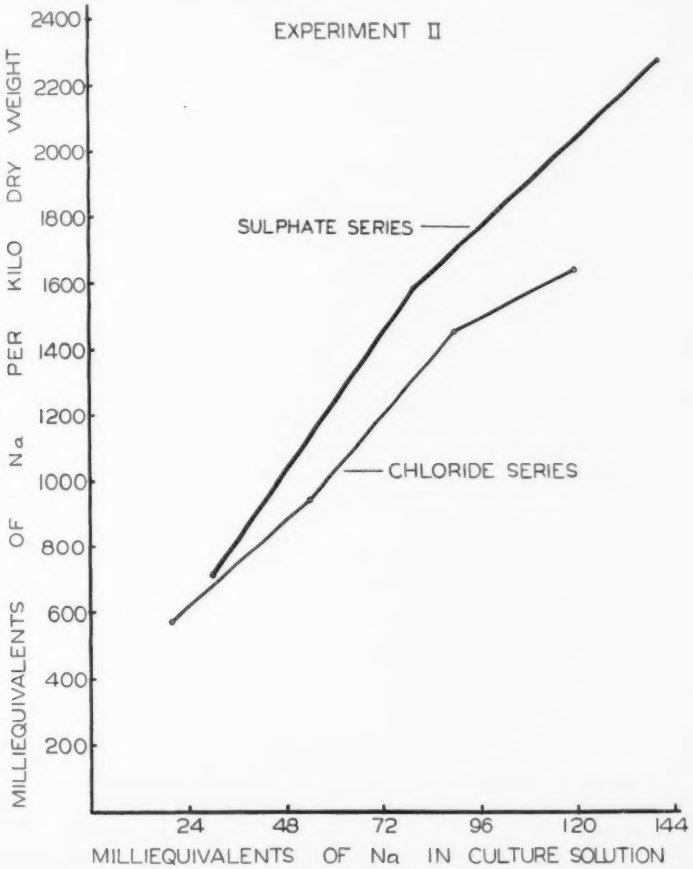

FIG. 4.—Experiment II. Sodium chloride and sodium sulphate series. Relation between concentration of sodium in culture solution and accumulation of sodium in tops.

STEM ANALYSIS.—The tomato stem has a dissected siphonostele consisting of three or four bicollateral bundles which at first are separated by broad medullary rays. As the stem matures the fascicular and interfascicular cambiums form a continuous cylinder, and a solid zone of secondary vascular tissue is produced. The mechanical tissues consist of a band of collenchyma lying centrad to a narrow

zone of chlorenchyma, and the outer and inner pericyclic fibers. A very small number of inner pericyclic fibers was differentiated (5).

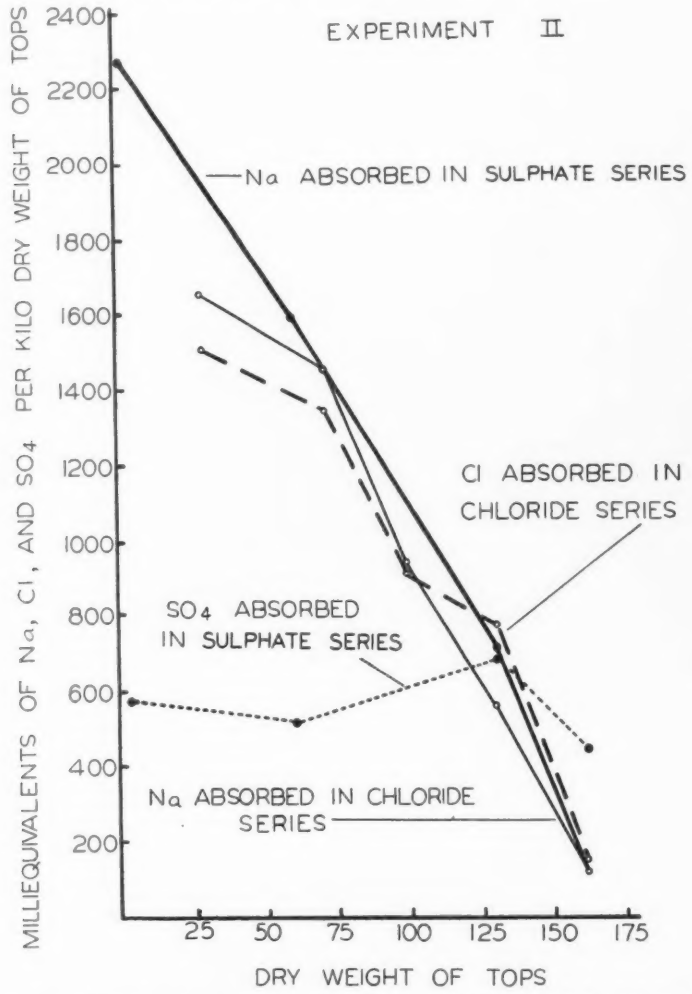

FIG. 5.—Experiment II. Sodium chloride and sodium sulphate series. Relation between accumulation of sodium, chloride, and sulphate ions and dry weight of tops.

With few exceptions the anatomical responses in corresponding cultures of the two experiments agreed with respect to their general character and differed only in degree. For this reason the results obtained in experiments I and II are dis-

cussed jointly for each series. Additional observations are included for the 6 atmosphere level in experiment II.

The smaller diameters of stems grown in solutions of high salt concentration were the result of differential reduction in their tissue systems. In general, the reduction of the vascular tissues was greater than that of the parenchymatous tis-

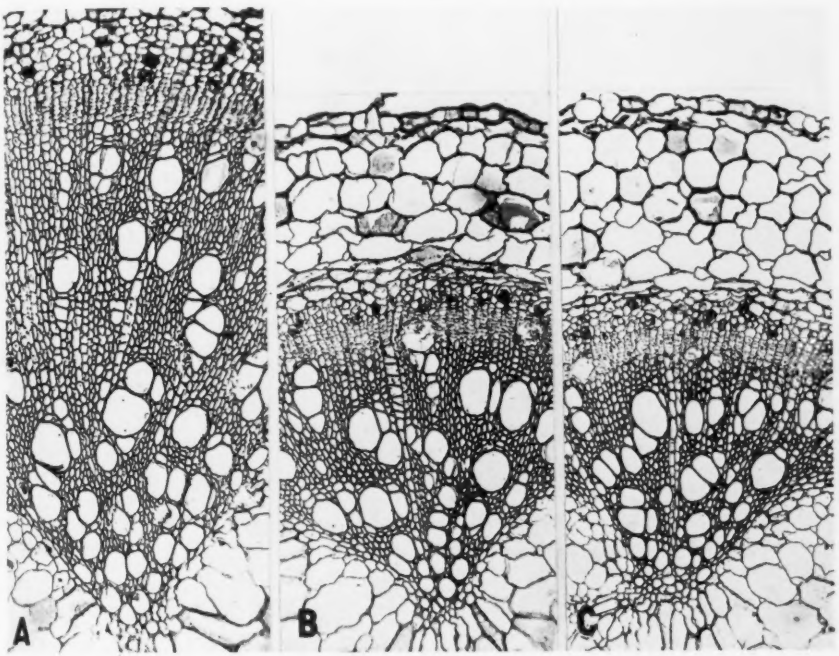

FIG. 6.—Experiment II. Base nutrient series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Sector of largest vascular bundle shown in each case. Osmotic concentrations in atmospheres: *A*, 0.5; *B*, 4.5; and *C*, 6.

sues of the cortex and pith. Measurements were taken of the width of cortex, radial dimension of the largest bundle, and width of the fascicular xylem, outer phloem, and interfascicular vascular tissue. The largest bundle in each transection was measured, as it was determined that its size constituted a reliable index of the total amount of fascicular tissue.

BASE NUTRIENT SERIES.—In both experiments there was a marked decrease in the amount of secondary vascular tissue at the high concentrations as compared with the 0.5 and 1.5 atmosphere cultures (fig. 6). Development of interfascicular vascular tissue followed the same general trend as that of the bundles. The reduc-

tion in the secondary (outer) phloem was proportionately greater than that of the xylem. The inner phloem was not measured, since little differential growth response occurred in the primary tissues (table 8).

TABLE 8

EXPERIMENTS I AND II: GROWTH MEASUREMENTS OF BASAL STEM* (IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	LARGEST BUNDLE		XYLEM		OUTER PHLOEM		INTERFASCICULAR VASCULAR TISSUE	
	I	II	I	II	I	II	I	II
	BASE NUTRIENT SERIES							
0.5	1910	1550	1550	1190	284	334	357	471
1.5	1900	1380	1610	1130	246	215	405	319
3.0	1530	1140	1250	968	191	152	349	258
4.5	1260	872	1070	723	136	114	243	212
6.0	.....	633	.....	532	.....	101	.....	117
	SODIUM CHLORIDE SERIES							
1.5	1900	1250	1610	1040	232	171	440	266
3.0	1600	942	1310	794	205	129	326	180
4.5	912	912	729	771	106	110	167	167
6.0	.....	765	.....	627	.....	106	.....	129
	SODIUM SULPHATE SERIES							
1.5	1650	1160	1350	934	223	197	395	238
3.0	1410	881	1120	714	182	136	326	238
4.5	737	471	592	380	98	60	174	115
6.0	.....	288	.....	259	.....	26	.....	80

* Radial dimensions, averages of four stems in each culture.

The rate of cambial activity was slightly higher at the 1.5 atmosphere level than at the 0.5, but was depressed at the high concentrations. As compared with the 1.5 atmosphere culture, the reduction in cambial rate at the 4.5 and 6 atmosphere levels was 25 per cent and 39 per cent, respectively. The total growth depression was a result of reduction in cell size as well as in number of cells formed. This cellular response was indicated by the decrease in the size of the xylem vessels at the high concentrations (table 9).

There was a marked decrease in the total amount of parenchyma in plants grown in the more concentrated culture solutions. With few exceptions, the per-

centage of medullary tissue increased with increasing osmotic concentration of the culture, while there was little change in the percentage of cortical tissue (table 10).

The differences in the areas of pith may be attributed mainly to variation in the size of the cells, which were larger in stems grown at the lower levels of concentration. The same condition prevailed in the cortex, but in some cases the tangential enlargement of the cortical cells was accompanied by radial divisions.

The collenchymatous cells of plants grown in the 4.5 and 6 atmosphere solutions were smaller than those in the less concentrated cultures, and the thickening

TABLE 9

EXPERIMENTS I AND II: ALL SERIES. MAXIMUM RADIAL DIMENSIONS OF SECONDARY XYLEM VESSELS (IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	BASE NUTRIENT		SODIUM CHLORIDE		SODIUM SULPHATE	
	I	II	I	II	I	II
0.5	150	126	.....	.....	.....	.....
1.5	152	139	141	132	133	126
3.0	126	123	135	114	99	89
4.5	117	109	108	92	69	45
6.0	.....	91	.....	85	.....	29

of the walls at the angles of the cells was more pronounced. The pericyclic fibers in plants grown at the higher osmotic concentrations were smaller in caliber and their secondary walls were proportionately thicker in comparison with the lumina of the cells.

SODIUM CHLORIDE SERIES.—As in the base nutrient series, there were significant reductions in the diameters of the stems grown in culture solutions with high osmotic concentrations as compared with those grown at the 1.5 atmosphere and control (0.5 atmosphere) levels (table 4).

The effect of high salt concentration on the development of secondary xylem and phloem was most pronounced in the more vigorously growing plants of experiment I; but in both experiments there was marked reduction in the amount of secondary fascicular xylem and phloem and in the development of interfascicular vascular tissue (fig. 7; table 8).

In this series, using the 1.5 atmosphere culture as a basis of comparison, the cambial activity was reduced approximately 25 per cent in the 4.5 and 34 per cent in the 6 atmosphere solutions. The effect of high salt concentration on the secondary xylem tissue was evidenced also by the reduction in the size of the vessels and increase in the thickness of their walls (table 9). The reduction in the amount of cortical and medullary parenchyma produced at the high levels of salt concentra-

tion was proportional to the reduction in the size of the stem. There was little variation in the percentage of the total area comprising the cortical and medullary parenchyma (table 10). The differentiation of collenchyma and pericyclic fibers was similar to that in the base nutrient series, but the thickness of the walls was more pronounced at the higher levels.

TABLE 10

EXPERIMENTS I AND II: TRANSECTIONAL AREAS OF STEM AT FIRST INTERNODE*

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	TOTAL AREA (SQ. MM.)		PERCENTAGE OF TOTAL AREA			
			CORTEX		PITH	
	I	II	I	II	I	II
	BASE NUTRIENT SERIES					
0.5	84	50	19	28	46	35
1.5	76	53	16	21	45	46
3.0	61	44	14	20	50	50
4.5	41	35	16	20	50	53
6.0	.....	28	.....	21	.....	57
	SODIUM CHLORIDE SERIES					
1.5	74	50	18	22	44	52
3.0	63	36	18	21	47	53
4.5	44	33	17	21	58	51
6.0	.....	23	.....	28	.....	46
	SODIUM SULPHATE SERIES					
1.5	63	44	19	20	45	50
3.0	41	28	21	26	41	45
4.5	15	8	25	43	43	33
6.0	.....	3	.....	64	.....	16

* Averages of four stems in each culture.

SODIUM SULPHATE SERIES.—The growth responses followed the same general pattern observed in the base nutrient and sodium chloride series, but the amount of growth depression was greater in this series at corresponding osmotic concentrations (table 4). At equal osmotic concentrations the total amount of vascular tissue was less in the sodium sulphate series than in the sodium chloride series (fig. 8). The reduction in the amount of secondary phloem was greater than that of secondary xylem, and this difference was more pronounced than in the base nu-

trient series. In the sodium sulphate series the amount of growth reduction was much greater between the 3 and 4.5 atmosphere levels than between the 1.5 and 3 atmosphere cultures (table 8).

The rate of cambial activity was much depressed at the 4.5 atmosphere level, being approximately 40 per cent less than that at the 1.5. In the 6 atmosphere culture there was no evidence of interfascicular activity in some of the stems, and the

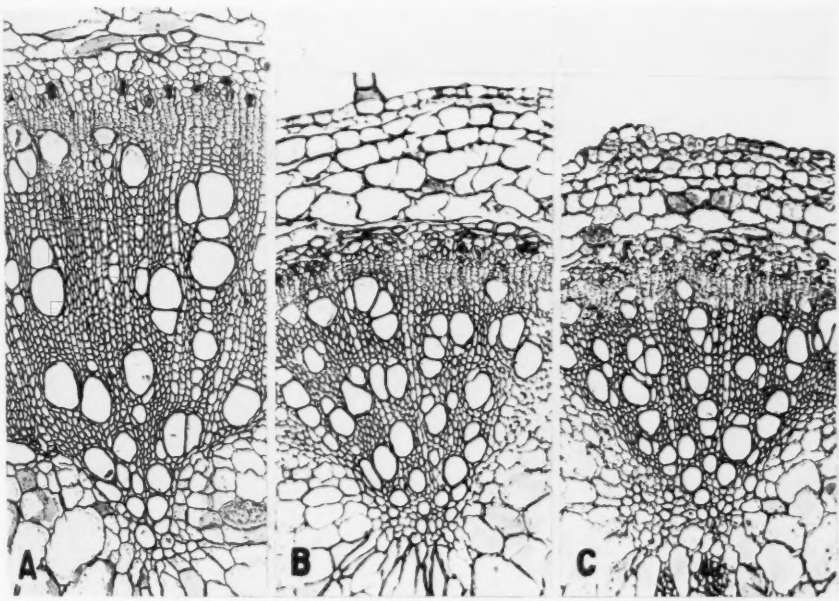

FIG. 7.—Experiment II. Sodium chloride series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Sector of largest vascular bundle shown in each case. Osmotic concentrations in atmospheres: *A*, 1.5; *B*, 4.5; and *C*, 6.

rate of activity of the fascicular cambium was about 60 per cent less than in the 1.5 atmosphere culture. As in the preceding series, there was a reduction in the size of the secondary xylem vessels and a relative increase in the thickness of the cell walls at the high concentrations (table 9).

In experiment I the proportion of cortical and medullary tissues to total transectional area increased slightly with increasing concentration, except at one level. In experiment II the percentage of cortical parenchyma increased markedly, while the medullary tissue decreased. The percentage of the total area comprised of parenchymatous tissue increased at the high concentrations, although the absolute amounts were much decreased (table 10).

There was pronounced accumulation of starch in the parenchymatous cells at the high concentrations. At the 4.5 and 6 atmosphere levels the cells of the endodermis and the pith cells adjacent to the vascular bundles were filled with starch grains. The cortical parenchyma and ray cells also contained more starch than at equivalent concentrations in either of the other series (fig. 8). At the high concentrations the cells of the collenchyma and the pericyclic fibers were smaller and the walls thicker in proportion to the size of the lumina.

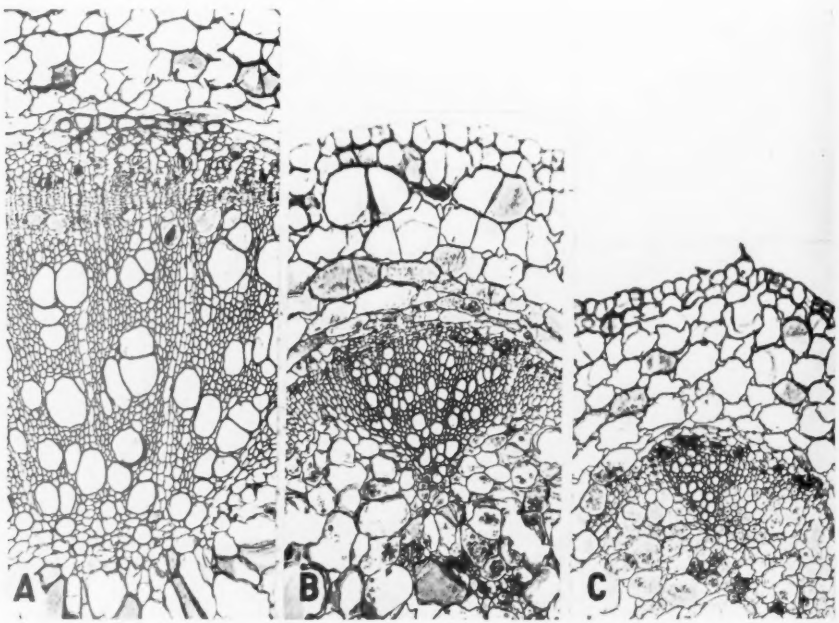

FIG. 8.—Experiment II. Sodium sulphate series. Transections of basal internodes showing progressive growth inhibition with increasing concentration of culture solution. Pronounced accumulation of starch indicated in *B* and *C*. Largest bundle shown in each case. Osmotic concentrations in atmospheres: *A*, 1.5; *B*, 4.5; and *C*, 6.

LEAF ANALYSIS

The blade of the leaflet is commonly thin, and the mesophyll consists of a single row of palisade cells and a spongy parenchyma of four or five layers of loosely arranged cells. The main vein projects prominently on the lower surface (fig. 9), and its bicollateral bundle is reinforced by abaxial and adaxial strands of collenchyma (5).

Transections of leaves were examined to determine the growth responses of the vascular tissue of the main vein and the tissues of the blade. The leaves were selected from the final harvest of experiment I and the first harvest of experi-

ment II. Leaves from the final harvest of experiment II were also examined, but these were not strictly comparable with those of the other two harvests, since they were 4 weeks older and had been subjected to less favorable environmental conditions during the additional period of growth. In all series, leaves from the 1.5 and 4.5 atmosphere cultures were examined, and additional studies were made from the 0.5 atmosphere culture in the base nutrient series and the 6 atmosphere cultures of experiment II.

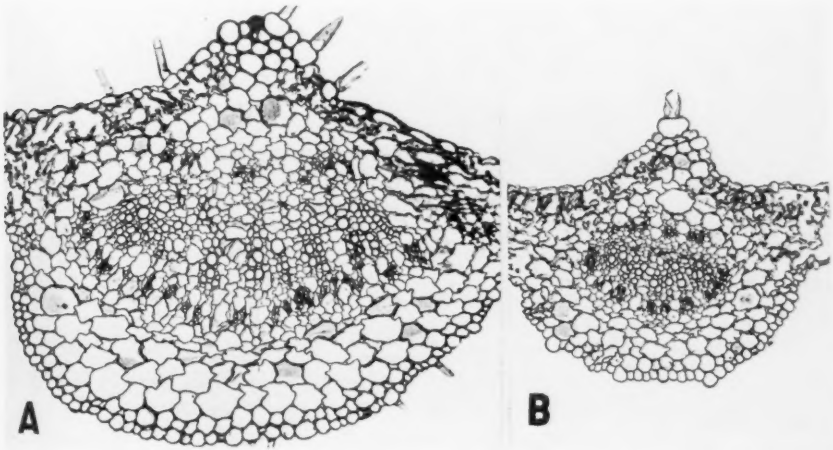

FIG. 9.—Experiment II. Sodium sulphate series. Transections of midribs of terminal leaflets showing growth inhibition at higher concentration of culture solution. Atmospheres osmotic concentration: *A*, 1.5; *B*, 4.5.

Representative leaves selected from the median portion of the plant axis were used for biometrical analysis. The height of the vascular bundle of the midrib of the terminal leaflet was used as an index of the amount of vascular tissue supplying the leaves. It provided a suitable basis of comparison with the radial dimensions of the vascular bundles of the stem. The average thickness of the lamina, the height of the palisade layer, and the thickness of the spongy parenchyma were also determined.

BASE NUTRIENT SERIES.—In this series the height of the vascular bundle of the main vein decreased at each successively higher concentration of the culture solution, resembling the growth depression of the bundles of the stem in this regard. In experiment II comparison of leaves from the 1.5 and 4.5 atmosphere cultures indicated a significant reduction in the thickness of the leaf of the latter, while in experiment I an increase occurred (table 11).

SODIUM CHLORIDE SERIES.—In both experiments there was a decrease in the vascular tissue of the midvein at the 4.5 as compared with the 1.5 atmosphere culture, and further growth depression was indicated at the 6 atmosphere level in experiment II. There was a significant increase in the thickness of the blade at the higher concentrations. The leaves were about 9 per cent thicker in the 4.5

TABLE 11

EXPERIMENTS I AND II: LEAF MEASUREMENTS (IN MICRONS)

CONCENTRATION OF SOLUTIONS (ATMOSPHERES)	MAIN BUNDLE HEIGHT		LAMINA THICKNESS		PALISADE HEIGHT		SPONGY PARENCHYMA THICKNESS	
	I	II	I	II	I	II	I	II
	BASE NUTRIENT SERIES							
0.5	.....	273	.....	187	.....	70	.....	92
1.5	300	237	158	177	61	57	73	84
4.5	253	223	174	151	61	56	87	72
6.0	.....	174	.....	139	.....	58	.....	64
	SODIUM CHLORIDE SERIES							
1.5								
4.5	314	246	176	156	67	58	87	70
6.0	228	190	192	170	71	68	101	74
	.....	177	.....	181	.....	76	.....	82
	SODIUM SULPHATE SERIES							
1.5								
4.5	311	266	158	163	48	58	83	73
6.0	228	145	206	175	62	66	116	85
	.....	148	.....	192	.....	69	.....	93

than in the 1.5 atmosphere cultures; and in experiment II those from the 6 atmosphere culture were 16 per cent thicker than leaves grown at 1.5 (table 11). Leaves from the final harvest of experiment II did not exhibit growth responses similar to those just recorded. The leaves grown at the 4.5 and 6 atmosphere levels were much thicker than those of the 1.5, but the leaves of the plants grown at 6 atmospheres were thinner than those of the 4.5.

SODIUM SULPHATE SERIES.—In both experiments there was a pronounced decrease in the size of the main bundle at the high concentrations (fig. 9). As compared with the 1.5 atmosphere culture, the growth at 6 was depressed about 45 per cent in the sodium sulphate series and 28 per cent in the sodium chloride.

There were significant increases in the thickness of the leaves at the 4.5 and 6 atmosphere levels, ranging from 9 per cent to 30 per cent. At the 4.5 atmosphere level the leaves of the sodium sulphate series were slightly thicker than those of the sodium chloride culture (table 11). At the high levels of salt concentration the relative density of the mesophyll tissues in the sodium chloride and sodium sulphate series appeared to be correlated with the degree of succulence. The lower percentage of dry matter in the tops of the sodium chloride plants as compared with those of the sodium sulphate and base nutrient series at equivalent concentration levels has been noted. In the leaves of plants grown in the high sodium sulphate cultures the palisade layer was much more compactly organized than in the leaves of the corresponding sodium chloride cultures (fig. 10).

Discussion

The significance of high concentrations of the soil solution in limiting the intake of water and soluble salts has been pointed out by MAXIMOV (**8**). BREAZEALE (**1**), growing wheat in solutions in which the concentration of nutrient salts ranged from 15 to 1550 p.p.m., concluded that the optimum concentration was approximately 155 p.p.m. SHIVE (**12**), using a three-salt solution, obtained best results at 1.75 atmosphere osmotic concentration, poorest ones at 0.1 atmosphere, and intermediate effects at 4. HOAGLAND (**6**) has defined optimum total concentration of a nutrient solution as "the least concentration giving a yield equal to any higher concentration." On that basis he found the optimum concentration to be 0.6 atmosphere and inhibitive concentrations at 2–2.5 atmospheres. TRELEASE (**13**) obtained optimum yields at 1.6 atmosphere, and found decreasing dry weight of tops with increasing concentration. NIGHTINGALE and FARNHAM (**11**) obtained best results with sweet peas at 0.5–1 atmosphere osmotic concentration.

In the present experiments with nutrient cultures at osmotic concentrations of 0.5, 1.5, 3, 4.5, and 6 atmospheres, maximum vegetative growth was obtained at 1.5 and a pronounced growth depression was noted at higher concentrations. No cultures were maintained at 1 atmosphere, and it is possible that for the solution used the optimum concentration as defined by HOAGLAND may lie somewhere between 0.5 and 1.5 atmosphere.

In cases where the high osmotic concentrations were obtained by the addition of sodium chloride or sodium sulphate to the base nutrient solution (0.5 atmosphere concentration), the growth depression at isosmotic concentrations was greater than in the base nutrient cultures. Comparisons of fresh and dry weights, gross morphology, and detailed anatomy indicated that the plants in the high sodium sulphate cultures were smaller than those in the corresponding cultures in the sodium chloride series. This suggests that there is an ionic influence which operates in addition to the effect of total salt concentration. EATON (**3**) has indicated

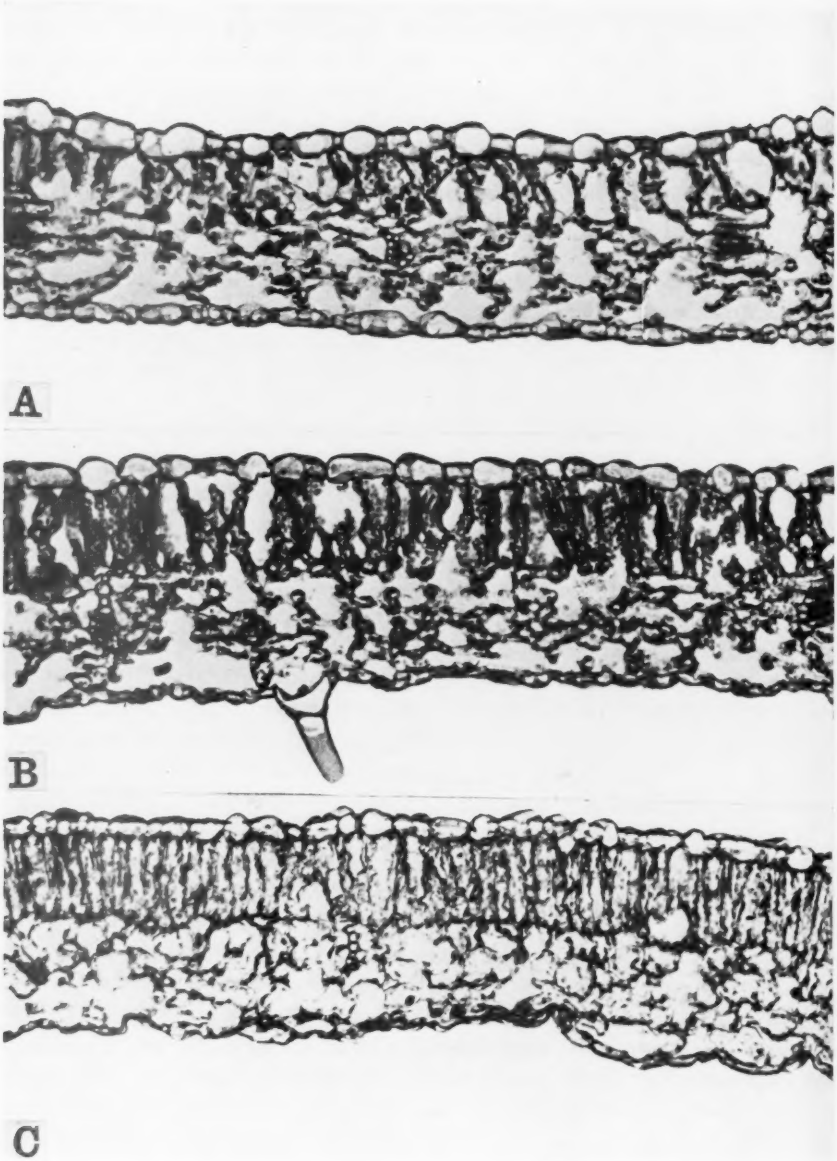

FIG. 10.—Transections of blades of terminal leaflets showing relative organization of palisade and spongy parenchyma: *A*, base nutrient culture, 1.5 atmosphere; *B*, sodium chloride culture, 4.5 atmospheres; *C*, sodium sulphate culture, 6 atmospheres.

a tendency for sulphate to be about half as toxic as chloride on a milliequivalent basis, but notes that "the tomato is out of line" in this regard.

In the sodium chloride series the percentage of dry matter increased very slightly or not at all with increasing concentration, while in the base nutrient series—and to a greater degree in the sodium sulphate series—there were marked increases in the percentage of dry matter, indicating a greater degree of succulence in the high chloride series.

The succulence of the leaves in the sodium chloride series corresponded with that observed by WUHRMANN (**16**), who found that the thickness and degree of succulence of the leaves of *Lepidium sativum* and *Nicotiana* could be modified by the addition of sodium chloride to nutrient solutions. VAN EIJK (**14**) investigated the effect of sodium chloride on the halophyte *Salicornia herbacea* and found optimum development with a 4/12 molar solution containing 2 per cent sodium chloride. He attributed the growth responses to the chloride ion rather than to the osmotic value of the solution or the concentration of the sodium ion, and concluded that cations do not affect succulence and that it is not induced by sulphate or nitrate ions. It seems possible, however, that the sodium ion may have an inhibitory effect on growth. At equal osmotic concentrations the solutions in the sodium sulphate series had a higher concentration of sodium than the sodium chloride cultures, and the accumulation of sodium by the plants showed a close relationship to the amount of sodium present in the solution, irrespective of the anion.

Growth depression may be the result of environmental factors which restrict the size of cells by accelerating their rate of maturation, or which inhibit the rate of activity of primary or secondary meristems. In the stems examined the number of primary cells at any given transectional level did not appear to be materially reduced by high salt concentration. The cells of the fundamental parenchyma were smaller, but the elements of the primary vascular tissue did not exhibit significant variation in size. The cells derived from the secondary meristems, especially the xylem elements, were smaller in size at the high concentrations.

In all series there was less cambial activity in plants grown in culture solutions with high osmotic concentrations than in those at the 1.5 atmosphere levels. Comparisons of cambial rate indicated that, at isosmotic concentrations, the rate was greatest in the base nutrient series, least in the sodium sulphate, and intermediate in the sodium chloride series. Between the 1.5 and 4.5 atmosphere levels the reduction in cambial rate ranged from 25 per cent to 42 per cent.

Starch accumulation may result from a number of factors. NIGHTINGALE (**9**) and FOSTER and TATMAN (**4**) have indicated the influence of temperature in this regard, and the latter have also called attention to the effects of variation in soil moisture. A number of investigators have shown that the carbohydrate metabo-

lism of the tomato plant is influenced by the supply of nutrient constituents, including ammonium, nitrate nitrogen, and potassium (**7, 10, 15**). Although no quantitative data were obtained, pronounced accumulation of starch in the storage parenchyma of the basal portion of the stem appeared to be correlated with high salt concentration. This was most evident in the high sodium sulphate cultures, in which the medullary, ray, and cortical parenchyma and the endodermal cells contained large reserves of starch.

Summary

Tomato plants were grown in three series of culture solutions: (*a*) a base nutrient series consisting of cultures adjusted to 0.5, 1.5, 3, 4.5, and 6 atmospheres osmotic concentration; (*b*) a sodium chloride series consisting of cultures containing base nutrient (0.5 atmosphere) plus sodium chloride to adjust the concentrations to 1.5, 3, 4.5, and 6 atmospheres osmotic concentration, respectively; and (*c*) a sodium sulphate series set up as in (*b*) but with sodium sulphate as the added salt instead of sodium chloride.

1. The concentration of the culture solution exerts an important influence on growth. In all series the growth of the plants as measured by height, diameter of stem, and dry weight was less at the high concentrations than at the control or 1.5 atmosphere levels. The greatest differences occurred in the sodium sulphate and the smallest in the base nutrient series.

2. The smaller diameters of stems at the high levels of salt concentration were correlated with significant differential reductions in the tissue systems. In general, the reduction of the vascular tissues, on the basis of percentage of total area, was greater than that of the parenchymatous tissue of the cortex and pith.

3. The reduction in the amount of vascular tissue at the high levels of salt concentration was related to more rapid maturation and consequent reduction in the size of the cells. The secondary xylem vessels were smaller in diameter at the high concentrations in all series, and the walls were thicker in proportion to the lumina of the vessel segments.

4. In the base nutrient and sodium sulphate series the proportion of secondary phloem to xylem was decreased at the high levels of salt concentration. In the sodium chloride series the proportionate amounts of secondary xylem and phloem remained approximately the same at all levels.

5. In all series the cells of the mechanical tissues were smaller in caliber and thicker walled in plants grown at high osmotic concentrations.

6. Cambial activity was inhibited by high concentrations of salts in all series. At isosmotic concentrations the cambial rate was greatest in the base nutrient and least in the sodium sulphate cultures.

7. The leaflets of plants grown at the high salt concentrations were significantly thicker in the sodium chloride and sodium sulphate series than in the base nutrient cultures.

8. The leaves of plants grown in high sodium chloride solutions were more succulent than those of the base nutrient and sodium sulphate series. This was correlated with the loose arrangement of the tissues of the mesophyll. In contrast, the palisade tissue in the leaves of plants grown in high sodium sulphate cultures was very compact.

9. The greater succulence of plants grown in high sodium chloride solutions was also indicated by the percentage of dry matter of tops. This remained essentially constant at all levels of concentration in the sodium chloride series, while it increased at the high concentrations in the base nutrient and sodium sulphate cultures.

10. Flower bud formation was retarded and probably reduced on plants grown in high sodium chloride solutions, and anthesis was delayed. There was a more pronounced effect in the high sodium sulphate cultures at the 4.5 atmosphere level, and no evident flower buds had developed up to the time of harvest.

11. High starch accumulation in the parenchymatous tissues of the basal stem was apparently correlated with high salt concentration in the culture solutions. This was most pronounced in the sodium sulphate cultures at 4.5 and 6 atmospheres osmotic concentration, but was also marked in the stems of plants grown at high sodium chloride levels.

12. Osmotic concentration of the sap increased in all series with increasing concentration of the culture solution.

13. With increasing concentration of the culture solutions there was increased accumulation of sodium and chloride ions in the tops of plants in the sodium chloride series. In the sodium sulphate series accumulation of the sodium ion increased as the concentration of the culture solution increased, but there was less correlation between the accumulation of the sulphate ion and the concentration of sulphate in the solutions.

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INFLUENCE OF PREPARATIVE PROCEDURE ON THE PURITY OF CHLOROPHYLL COMPONENTS AS SHOWN BY ABSORPTION SPECTRA

F. P. ZSCHEILE AND C. L. COMAR[1]

(WITH FOUR FIGURES)

Introduction

The lack of agreement on the quantitative expression of the absorption spectra of chlorophylls *a* and *b* has been the source of considerable confusion. This has been ably discussed recently by MACKINNEY (**7**). The causes of these difficulties may be broadly classified into two groups, those resulting from the use of different spectro-photometric methods and those arising from the presence of accompanying impurities or decomposition products. As will be shown later, the latter group is perhaps of more importance because of the extreme lability of isolated chlorophyll. The high sensitivity of the spectro-photoelectric method makes it possible to detect changes which cannot be followed by other methods. The establishment of accurate absorption values for the individual chlorophyll components is essential for quantitative treatment of the relations of light to chlorophyll systems, as in photosynthesis, and is indispensable for spectroscopic analysis of pigment mixtures.

While reinvestigating the absorption spectra of chlorophylls *a* and *b* in ether solution, it was observed that significant changes were caused by variations in preparative procedure. Further study of these changes led to certain modifications of existing methods which seemed necessary to avoid decomposition. The present method will be described in some detail, because neglect of certain precautions—one of which has not been observed in previously described methods for pigment isolation—may lead to an amount of decomposition which may alter the absorption values considerably. Such slight changes in composition cannot be followed reliably by elementary analysis.

Experimentation

Numerous variations in procedure have been tried and the following method has been found to yield chlorophyll components with consistent spectroscopic properties. Certain general features of the WILLSTÄTTER-STOLL (**10**) method have been retained. Both components are separated and purified by adsorption.

[1] Purdue Research Foundation Fellow.

Preparation of chlorophyll a

A semi-darkened room is desirable for all preparative procedures. About 1 kg. of clean fresh green leaves is disintegrated in a Waring Blendor (1), in the presence of cool acetone (about 20° C.) to which a small amount of calcium carbonate has been added. One hundred gm. of leaf material is conveniently extracted in 400 cc. of acetone; usually 3–5 minutes in the Blendor per batch is sufficient for preparative extraction. These conditions may be varied somewhat, depending on the type of material used and the degree of extraction desired. Usually one extraction leaves the cell fragments almost colorless after filtration and washing.

The mixture is filtered by suction and the resulting acetone extract is added carefully to a solution containing 1 liter of petroleum ether[2] and 100 cc. of acetone in a 4-liter glass separatory funnel. The filtrate from each batch is added individually. After each third addition, the solution is rotated gently and distilled water carefully added until two distinct layers are formed. (It is advisable that a small amount of calcium carbonate be added to all distilled water used for washing purposes.) Most of the acetone and water-soluble impurities are removed in the aqueous layer, which is discarded before further addition of acetone filtrates.

The petroleum ether solution is scrubbed through distilled water. This is done by allowing the solution to flow by gravity through a narrow tube submerged 5–10 cm. below the surface of the water (7), contained in a 4-liter separatory funnel. The solution is then washed five or more times with 200 cc. of 80% methyl alcohol. Before use, the methyl alcohol is saturated with petroleum ether and 0.01 gm. of oxalic acid per liter added to prevent allomerization. The last washing should be a light greenish yellow. The solution is now scrubbed through distilled water until precipitation of the chlorophyll occurs; three or four washings usually suffice and the time involved in this step is 1–2 hours.

The chlorophyll suspension is dried over anhydrous sodium sulphate and filtered on 3-cm. layer of powdered sucrose (ordinary powdered sugar[3] is used). This layer is made on a Buchner funnel 14 cm. in diameter and 5.5 cm. deep. The sucrose is wet with petroleum ether and suction applied; the sugar is pressed down and, when dry, rubbed to form a smooth hard surface. The precipitated pigment is washed exhaustively with petroleum ether to remove carotenoids. The pigment layer is mechanically removed, while still moist, and extracted with a maximum of 60 cc. of ether.[4] The resulting solution contains crude chlorophyll. This step requires 30–50 minutes.

The ether solution of crude chlorophyll is made up to contain 70% petroleum ether and 30% ether. This solution is adsorbed on sucrose, the column dimensions being 5 cm. in diameter and about 35 cm. in length. When used for adsorption,

[2] All petroleum ether used had a b.p. of 35°–45° C.

[3] C and H confectioner's powdered sugar (contains 3% starch).

[4] Ether designates diethyl ether.

the powdered sugar is first dried at 90° C. in vacuum and stored in air-tight bottles. Under normal conditions only two zones are observed: a green band 4–10 cm. in length and below it a contiguous blue zone 2–6 cm. in length. The time required for this separation is only about 40 minutes, since it has not been found necessary to develop the chromatogram by washing. The green layer is mechanically removed and may be used for the subsequent isolation of chlorophyll *b*, although it was found more convenient to make a separate extraction for the preparation of the *b* component. The walls of the column are then cleaned with ether-soaked cotton to avoid any minute contamination of the chlorophyll *a* with the *b* component. The center half of the blue layer is removed and immediately stirred into ether, the top and bottom portions being discarded. The resulting suspension is filtered on a Buchner funnel and the pigment thus eluted. The ether solution is washed thoroughly with distilled water and dried over sodium sulphate. The solution is filtered through a fine sintered glass plate, and the pigment is then ready for spectroscopic study. The time required for this entire preparation should not exceed 5–6 hours.

Preparation of chlorophyll b

The procedure described for chlorophyll *a* is followed in detail up to and including the adsorption of the pigments on sucrose. In the isolation of the *b* component, however, it is necessary to wash the column exhaustively; 1–1.5 liters of fresh solvent (70% petroleum ether, 30% ether) are used. After the washing, the column consists of a 5–7 cm. layer of colorless adsorbent, below which is a 23–27 cm. green zone, which in turn is followed by the blue band of component *a*. The center portion of the green zone is mechanically removed, taking all precautions to avoid contamination, and is stirred immediately into ether, filtered, and eluted.

This solution is made up to contain 70% petroleum ether and 30% ether and is readsorbed on sucrose. After washing as indicated, the green zone is usually separated from the blue by about 1 cm. of colorless adsorbent. The center portion of the green zone is again removed and the pigment eluted. The pigment solution is again adjusted to a concentration of 70% petroleum ether and adsorbed a third time. This chromatogram should consist of a single homogeneous zone. The final pigment solution is prepared in precisely the manner described for chlorophyll *a*. Since each column requires about 2 hours, the total time required for the isolation of the *b* component from plant material is 11–13 hours. Ordinarily one worker will require 2 working days for this preparation and for spectroscopic observations. Ether solutions may be kept over night at 0° C.

The solvents were purified as described by Zscheile (**13**). In some cases the ether was purified by distillation after standing over solid potassium hydroxide. The spectroscopic results were the same as when sodium was used.

Absorption methods

The absorption spectra were determined by a spectro-photoelectric method similar to that described by Hogness, Zscheile, and Sidwell (4). The most important difference is that in our optical system a Large Müller-Hilger Universal Double Monochromator with crystal quartz optics is employed instead of a Zeiss Fixed-arm Spectroscope (with deviation of 90°) with glass optics. A figured crystal quartz lens, corrected for spherical aberration, is used instead of the simple biconvex crystal quartz condensing lens 1 (4, fig. 2). A crystal quartz field lens is mounted directly in front of slit 1. No other lenses are used to focus the source radiation upon the slit. Otherwise the two sets of equipment are very similar, except for certain changes made for greater convenience or stability. The entire set-up will be described in full at a later time.

All three slits of the double monochromator were maintained at equal widths and the exit slit was confined to a length of 6 mm. by a diaphragm. The dispersion of this instrument with quartz optics is almost equal to that of the Zeiss instrument with glass optics from 4000–5600 A and is greater from 5600–7000 A. The dispersion, expressed in A per millimeter, is as follows: 63 at 4000 A, 168 at 5500 A, and 290 at 7000 A. Measurements were made at intervals of 20–40 A over most of the spectrum and at 10-A intervals over the higher maxima. The region from 3800 to 6900 A was studied.

The cell[5] length was 1.0003 cm., and the concentration was adjusted to keep the $\log_{10} \frac{I_0}{I}$ values between 0.200 and 0.800. The specific absorption coefficient a is defined as follows:

$$a = \frac{\log_{10} \frac{I_0}{I}}{cl}.$$

I_0 = intensity of light transmitted by solvent-filled cell.
I = intensity of light transmitted by solution-filled cell.
c = concentration in gm. per liter.
l = thickness of solution layer in cm.

The following technique is used to obtain the concentration of an ether solution of chlorophyll from a 10-cc. aliquot of that solution. A clean, tared 10-cc. volumetric flask is filled to the mark with the solution. The ether is evaporated under a water pump vacuum, care being taken to avoid spattering. After the ether is removed the flask is kept at 103° C. for an hour, allowed to cool for 30 minutes in a

[5] Fused Pyrex absorption cells (American Instrument Co., Style DX, Class 3 Tolerance) with ground stoppers, were used.

desiccator, and weighed on a microbalance. It is advisable to tare the flask by using pure ether and following the procedure already described. When the concentration is 0.2 gm. per liter or more, the error is $\pm 0.4\%$ as checked against known solutions prepared from weighed samples of dried chlorophyll. In most cases the average error of three determinations is less than $\pm 0.3\%$. For lower concentrations, or when greater accuracy is desired, 25-cc. volumetric flasks may be used.

Results

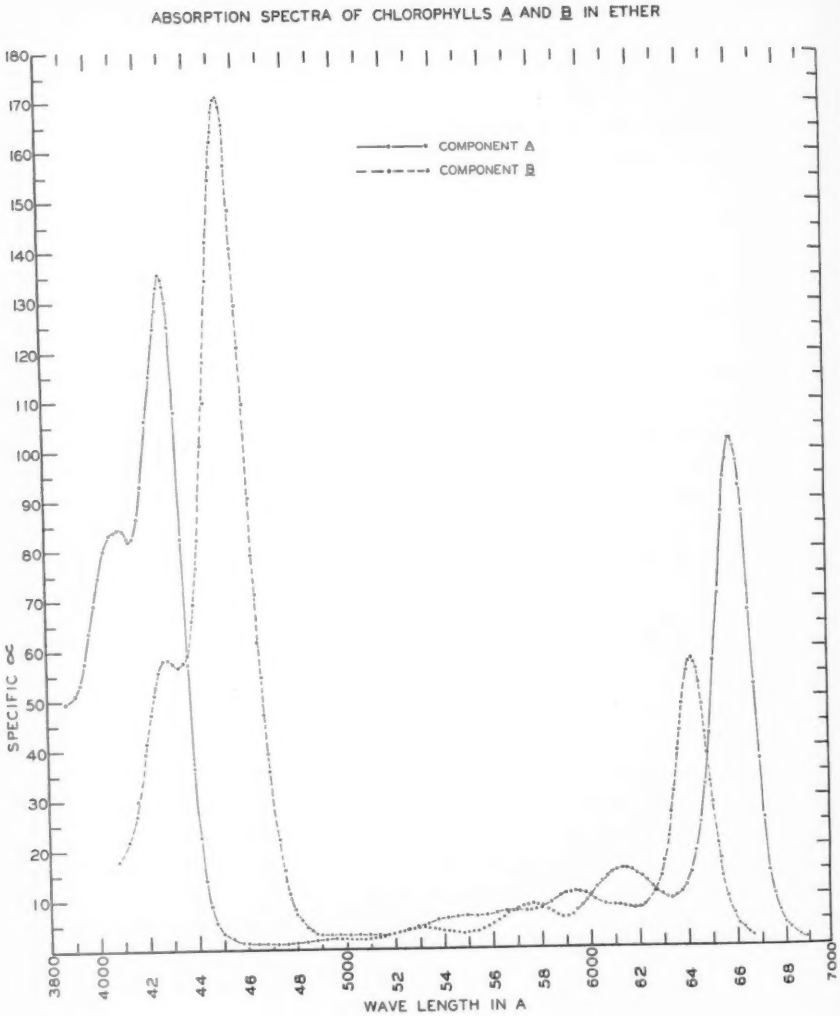
Absorption spectra

The absorption spectra of chlorophylls *a* and *b* in ether solution are presented in figure 1. In figure 2 the regions of low absorption between 4400 and 6400 A are plotted on an enlarged scale to facilitate later discussion; the accuracy of presentation is then more comparable with that of the measurements. The experimental points are chosen from two or more complete curves of different preparations to produce a representative curve. Tables 1 and 2 present the numerical values of absorption coefficients at maxima and minima for numerous preparations of chlorophyll. Typical values for concentrations and spectral regions isolated are representative of the group but are not all descriptive of the same preparation.

The ultraviolet absorption spectrum has been investigated in a very preliminary way. No detail of special interest was observed.

We have used diethyl ether as a solvent for spectroscopic observations, chiefly because the sucrose from the adsorption column can be completely removed from ether by washing with water. When acetone is used for direct elution from the adsorbent, a small amount of sucrose remains in solution and causes large errors in the weight determination of the chlorophyll. Attempts to correct for sucrose content by the use of acetone saturated with sucrose or by removal of sucrose quantitatively by freezing methods were unsuccessful. Moreover, data from more laboratories are available for ether solutions than for other solvents and we have experienced no difficulty in the use of ether. All the solutions, solvent, and glassware employed for dilutions were maintained at 25° C., and the absorption cell chamber was thermostated at 25° C.

In figures 3 and 4 the spectra of the individual components are compared with those of the products formed from the components by acid. The "pheophytin" was prepared by the addition of approximately 0.1 cc. of concentrated hydrochloric acid to the pure chlorophyll component in approximately 50 cc. of ether solution. The absorption measurements were made after the solution had remained 1–3 days at room temperature in the dark. The concentration was calculated from that of the original pure chlorophyll solution, assuming that two hydrogens replace the magnesium in the molecule. The maxima of these curves agree


ABSORPTION SPECTRA OF CHLOROPHYLLS A AND B IN ETHER
COMPONENT A
COMPONENT B
SPECIFIC α
WAVE LENGTH IN A
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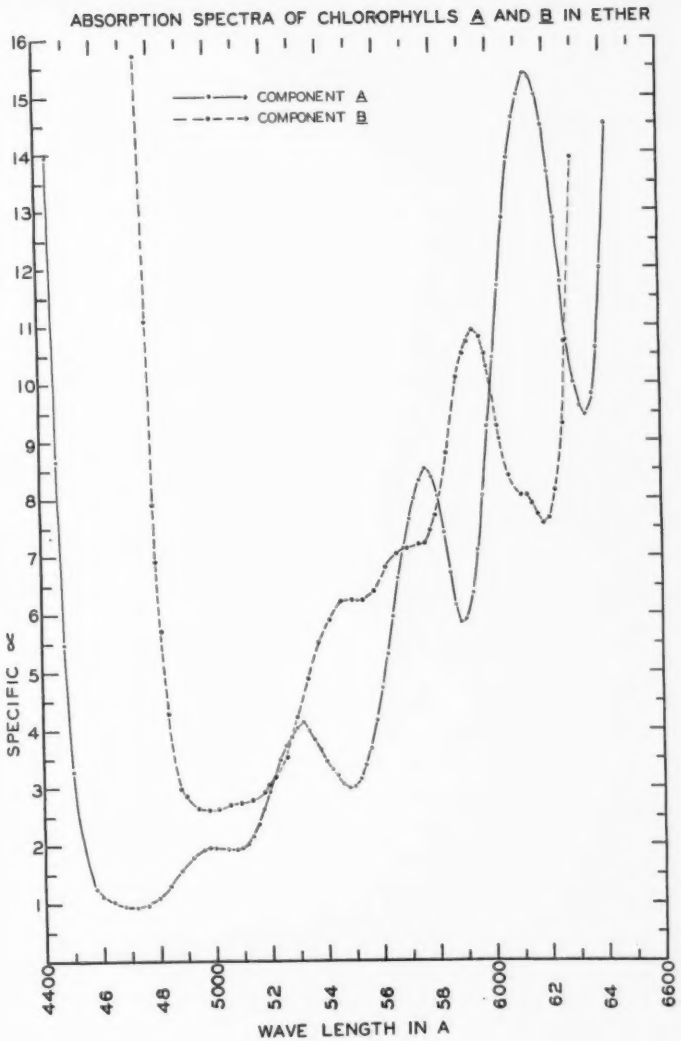
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
3800
4000
42
44
46
48
5000
52
54
56
58
6000
62
64
66
68
7000

FIG. 1


ABSORPTION SPECTRA OF CHLOROPHYLLS A AND B IN ETHER
COMPONENT A
COMPONENT B
SPECIFIC α
WAVE LENGTH IN A
16
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12
11
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8
7
6
5
4
3
2
1
4400
46
48
5000
52
54
56
58
6000
62
64
6600

FIG. 2

TABLE 1

ABSORPTION SPECTRA OF CHLOROPHYLL *a* IN ETHER SOLUTION

WAVE LENGTH A	SPECIFIC ABSORPTION COEFFICIENTS						AVERAGE	MEAN DEVIATION (%)	TYPICAL CONCENTRATION PER LITER (GM.)	TYPICAL SPECTRAL REGION ISOLATED (A) (4)
	1 SPINACH	2 SPINACH	3 CORN	4 SPINACH	5 SPINACH	6 SPINACH				
Maxima:										
6600	103.5	101.6	103.5	101.8	100.2	101.8	102.1	0.94	0.003	30
6140	15.7	15.3	15.6	15.6	15.4	15.3	15.5	0.98	0.030	26
5760	8.44	8.56	8.50	8.56	8.50	8.50	8.51	0.39	0.040	23
5320	4.22	4.19	4.18	4.16	4.03	4.03	4.14	1.34	0.120	18
5050 (pheophytin *a* maximum)	1.96	1.97	1.94	1.93	1.97	1.94	1.95	0.77	0.200	16
4290	139†	131	137	138	133	135	135	1.63	0.003	10
4100	89.0†	83.8	86.5	85.5	85.7	84.3	85.2	1.03	0.003	9
Minima:										
6330	9.32	9.40	9.46	9.80	9.76	9.45	9.53	1.73	0.040	28
5890	5.92	5.90	5.76	6.00	6.12	5.85	5.93	1.54	0.050	24
5480	2.96	3.05	3.02	3.17	3.01	2.99	3.03	1.67	0.150	20
4720	1.60†	0.956	0.928	0.923	0.929	1.27†	0.934	1.18	0.230	13
R_a*	52.8	51.6	53.3	52.7	51.0	52.4	.....	.....	.....	.....

* $R_a = \frac{a6600}{a5050}$.

† These values excluded from averages because of carotenoid content.

TABLE 2

ABSORPTION SPECTRA OF CHLOROPHYLL *b* IN ETHER SOLUTION

WAVE LENGTH A	SPECIFIC ABSORPTION COEFFICIENTS			AVERAGE	MEAN DEVIATION (%)	TYPICAL CONCENTRATION PER LITER (GM.)	TYPICAL SPECTRAL REGION ISOLATED (A)
	1 BARLEY	2 SPINACH	3 SPINACH				
Maxima:							
6425	57.6	55.4	57.5	56.8	1.71	0.008	38
5940	11.1	10.6	10.9	10.9	1.83	0.040	32
5500	6.27	6.10	6.24	6.20	1.13	0.040	27
4530	173	169	171	171	0.78	0.003	15
4300	59.1	58.2	58.1	58.5	0.74	0.008	13
Minima:							
6180	7.74	7.54	7.55	7.61	1.14	0.040	35
5200 (pheophytin *b* maximum)	3.15	3.02	3.07	3.08	1.51	0.180	23
5100	2.68	2.64	2.73	2.68	1.12	0.180	22
R_b*	18.3	18.3	18.7	.....	.....	.....	.....

* $R_b = \frac{a6425}{a5200}$.

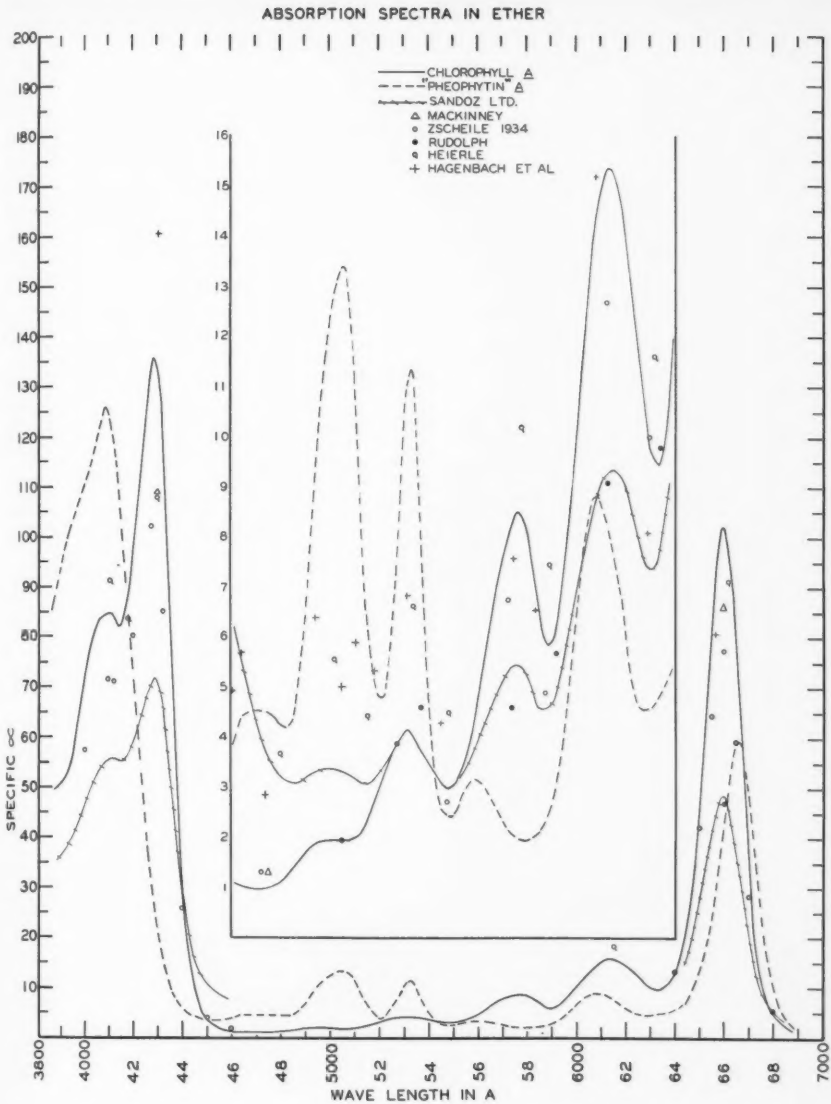
ABSORPTION SPECTRA IN ETHER
CHLOROPHYLL A
PHEOPHYTIN A
SANDOZ LTD.
MACKINNEY
ZSCHEILE 1934
RUDOLPH
HEIERLE
HAGENBACH ET AL
SPECIFIC ∝
WAVE LENGTH IN A
3800
4000
42
44
46
48
5000
52
54
56
58
6000
62
64
66
68
7000

FIG. 3

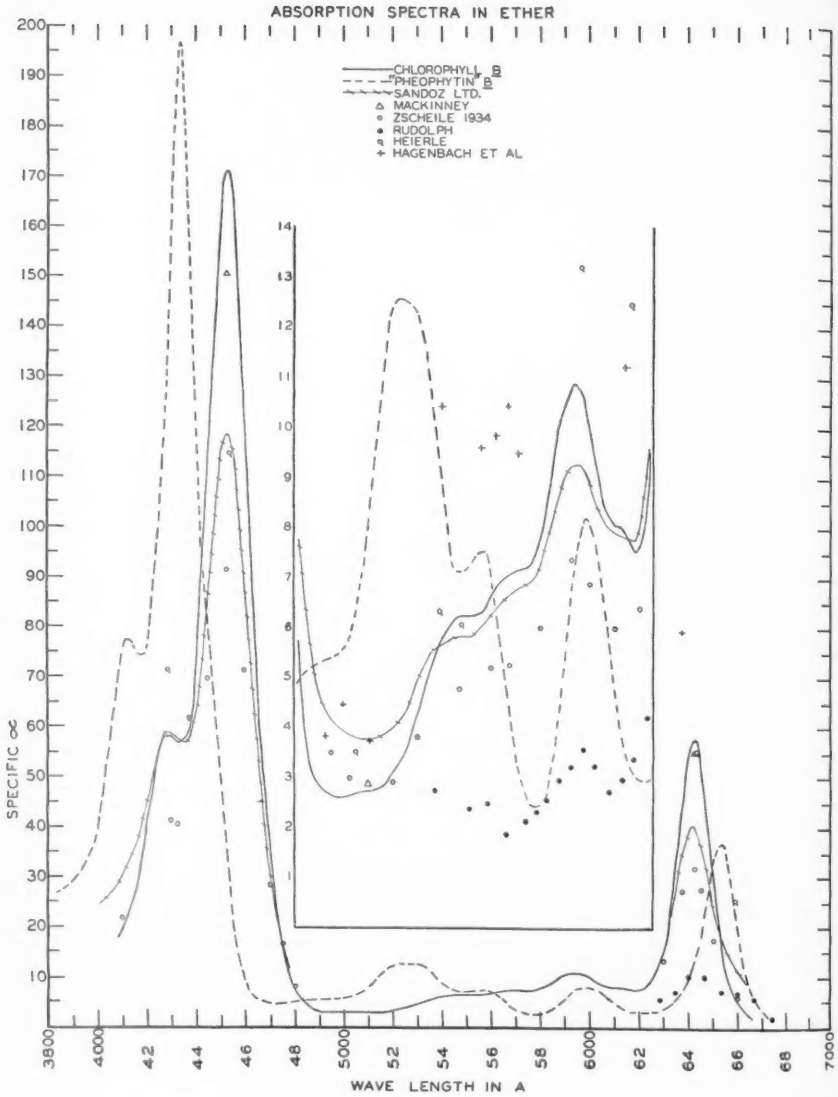
ABSORPTION SPECTRA IN ETHER
CHLOROPHYLL B
"PHEOPHYTIN" B
SANDOZ LTD.
MACKINNEY
ZSCHEILE 1934
RUDOLPH
HEIERLE
HAGENBACH ET AL
SPECIFIC ∝
WAVE LENGTH IN A

FIG. 4

in wave length with those ascribed to pheophytin in the literature. These curves are not presented as those of pure pheophytin, however, since different preparations had absorption coefficients which differed by 2–15% at certain wave lengths. The most representative curves are plotted. For the purposes of this paper, these differences are unimportant.

Figures 3 and 4 also contain the absorption spectra of chlorophyll components *a* and *b*, which were purchased from Sandoz, Ltd., Basle, Switzerland. They are designated by the manufacturer as "chromatographically pure chlorophyll preparations for scientific purposes," purified by adsorption on sugar. The preparations were made in September, 1939, from the leaves of the stinging nettle (*Urtica dioica*). The spectra were determined on weighed samples in September, 1940. In addition, other absorption values for chlorophyll components in ether solution, which have come to the writers' attention, are included for comparative purposes.

Spectroscopic criteria of purity

Since chlorophyll tends to lose its magnesium relatively easily, it is of interest to study the spectra of pheophytin. It was noted that the absorption values of all our chlorophyll *a* preparations which had undergone small amounts of degradation occurred between the chlorophyll and pheophytin values, especially for wave lengths above 4700 A. It is evident from the nature of the curves that initial degradation appears to be similar to pheophytin formation. Hubert's data (**5**) on the effect of light on chlorophyll also show this trend.

It is clear (figs. 3, 4) that small amounts of pheophytin *a* in chlorophyll *a* may be most easily detected by a study of the spectral regions near 6600 and 5050 A. In the case of *b*, the wave lengths are 6425 and 5200 A. Thus the ratio $\frac{a6600}{a5050}$ may be used as a sensitive criterion for the usual forms of degradation in chlorophyll *a*. This ratio will hereafter be designated as R_a. R_b is the ratio $\frac{a6425}{a5200}$.

In experiments with calcium carbonate as an adsorbent, it was noticed that chlorophyll *a*, which had been dried by evaporation with a water pump and then stored in vacuum over night at 0° C., formed an olive green ether solution instead of the original blue. This was indicative of pheophytin formation, and raised the question as to whether the same thing might not occur when other adsorbents were used, but to a less degree, so that the change could not be observed visually. This change was studied spectroscopically. Willstätter and Stoll (**10**) observed a change of spectroscopic properties when certain chlorophyll derivatives were dried from ether solution. Our samples designated as "dried" were evaporated to dryness in beakers with a water pump and immediately placed in a vacuum of about 20 μ. All samples were kept at 0–5° C. and not exposed to light. The glass-

ware was washed in acid dichromate solution, followed by tri-sodium phosphate to avoid the possibility of degradation due to residual acid on the walls. The following data are chosen as representative of many experiments showing the same trends.

Sample and treatment	R_a
I. Chlorophyll *a* prepared by adsorption on calcium carbonate	42.3
A. Preparation I stored in solution over night	38.7
1. Preparation IA stored in solution over night	33.8
2. Preparation IA stored dry over night	19.7
II. Chlorophyll *a* prepared by adsorption on sucrose	50.8
A. Preparation II in solution for 8 days	49.1
B. Preparation II dry for 8 days	46.9
III. Chlorophyll *a* prepared by adsorption on sucrose	52.4
A. Preparation III in solution for 13 days	51.3
B. Preparation III dried with oxygen-free nitrogen, allowed no contact with air, and stored for 13 days	42.8
IV. Chlorophyll *a* prepared by adsorption on sucrose and mistreated by drying and storage under laboratory conditions	32.9
A. Preparation IV in solution over night	33.0
B. Preparation IV dry over night	30.1
C. Preparation IV stored dry on powdered glass over night	23.4
	R_b
V. Chlorophyll *b* prepared by adsorption on sucrose	18.9
A. Preparation V in solution for 5 days	17.7
1. Preparation VA in solution for total of 9 days	16.6
B. Preparation V dry for 5 days	13.9
1. Preparation VB redried and stored for 4 more days	4.4

It is evident that the adsorbent may influence the stability of the final solution. The drying effect is independent of the presence of oxygen. Redrying and increased contact with glass surfaces apparently increase the amount of degradation. All samples show a positive phase test and, in the case of chlorophyll *a*, solutions having a ratio as low as 30 cannot be distinguished visually from those with ratios of 51 to 53.

Other criteria of purity

The phase test was positive on all preparations; the transient color was yellow for component *a* and red for component *b*.

The absence of hydrolytic loss of phytol is indicated by the fact that color was not extracted from ether solutions by 22% HCl. This is confirmed by an analysis for total nitrogen which gave a value of 6.18% nitrogen in chlorophyll *a*. The theoretical value is 6.28%. In no case has difficulty been experienced due to hydrolysis, nor is there any evidence that it had occurred.

Discussion

Preparation

Exposure of the pigments to light, heat, drying, or acid must be avoided during the preparation. Small amounts of calcium carbonate are added to the acetone used in extraction (to neutralize plant acids which may be liberated) and also to all distilled water used in washing procedures.

The extreme sensitivity of chlorophyll solutions to light is shown by an experiment in which a dilute ether solution of chlorophyll *a* in the absorption cell was exposed to ordinary room light (northern exposure) for 30 minutes. The absorption value at 4290 A decreased 14% during this time. The original stock solution, kept in the dark 3 hours at room temperature, showed no decrease. This indicates the necessity for elimination of all possible light exposure at all times.

Fresh leaves are used in order to avoid any possible pigment degradation due to drying. Acetone has been found satisfactory for extraction. Ice may be added directly to the mixture in the Waring Blendor to counteract heat generated by the Blendor or effects of excessively warm weather.

The pigments may be transferred to petroleum ether after having been in contact with acetone for only 5–7 minutes; this minimizes the possible loss of phytol due to enzyme action (**7**). The methyl alcohol washing serves to eliminate most of the xanthophylls and, although it causes considerable loss of chlorophyll *b*, the yield is always sufficient for quantitative spectroscopic studies.

The scrubbing method, suggested by Mackinney (**7**), greatly shortens the time required for precipitation. The chlorophyll suspension is filtered on sucrose rather than talc, since the former may be easily washed out of the ether solution and there is evidence that contact with talc may cause degradation of the pigment. Winterstein and Schön (**12**) also observed this cause of degradation. Experiment has shown that a second washing with methyl alcohol, reprecipitation, and rewashing with petroleum ether usually do not increase the purity of the preparation so far as contamination with carotenoids is concerned. It was observed that removal of carotenoids is essential for successful separation of *a* and *b* components on sucrose.

Sucrose was chosen for separation of the components by adsorption because of the following facts: (*a*) we have found that sucrose does not induce pigment degradation, which is not the case for $CaCO_3$, $Ca(OH)_2$, MgO, and at least for certain brands of talc; (*b*) under our conditions chlorophyll *b* may be obtained as a discrete zone on the second column—Mackinney (**7**) obtained this result with inulin; (*c*) sucrose is readily removed from ether solutions by washing with water; and (*d*) the material is inexpensive and readily available.

When old chlorotic leaves are used, or when pigment degradation has occurred,

a dark layer (probably pheophytin) is found below the *a* zone on the column. When all precautions have been observed, however, no indication of degradation is evident either below the *a* or above the *b* band. According to WILSON (11), the uppermost color zone of a chromatogram contains some of each solute initially present; this has been confirmed by the present observations. Thus in a column in which the zones are contiguous, all except the lowest would be expected to contain more than one substance. If pheophytin formation is avoided, chlorophyll *a* will be the lower zone and there will be no need for exhaustive washing of the chromatogram when this component is desired. The difficulty and uncertainty in the separation of pheophytin *a* from chlorophyll *a* by adsorption emphasize the need for the prevention of pigment degradation during the preparation. To obtain chlorophyll *b*, the column must be washed exhaustively. Readsorption is always necessary, and if the amount of *a* is sufficiently low, it is possible to obtain the *a* and *b* bands as discrete zones. The third chromatogram should consist of a single *b* band and is a check on the complete removal of *a*.

The spectroscopic observations were made immediately after preparation of the final ether solution and before the weight data were available.

PRECISION AND RELIABILITY OF ABSORPTION SPECTRA

Under present operating conditions, the precision error of measurement has been found to be $\pm 0.5\%$ for $\log_{10} \frac{I_0}{I}$ values of 0.2–0.8, the working range given by HOGNESS, ZSCHEILE, and SIDWELL (4) for small values of $\frac{\Delta I}{I_0}$. It has also been demonstrated that total precision errors, including weight and volumetric technique, do not exceed $\pm 0.6\%$. The uncertainty between preparations is of course larger than this. As may be seen from the data, the maximum mean deviation from the average for all wave lengths listed is 1.83%; however, the average mean deviation is 1.22%. There is no significantly greater error in regions of high absorption than in regions of low absorption.

There are other factors which might influence absolute values obtained by this method. The spectral region isolated by the monochromator, as defined earlier (4), may influence the absorption coefficients obtained, depending on the wave length used and the nature of the absorption curve. The absorption value tends to be an average for all the wave lengths of radiation included in the spectral region employed. Values at narrow maxima or minima are therefore more influenced by variation of region isolated. The blue and red maxima of the chlorophylls are examples of this.

The absorption values for chlorophyll *a* solution have been studied as a function of spectral region isolated at the more critical wave lengths. Constant values were obtained under the following conditions:

4290 A (maximum)	slits 0.03–0.25 mm.	spectral region 5– 40 A
4720 A (minimum)	slits 0.04–0.5 mm.	spectral region 9–110 A
6600 A (maximum)	slits 0.02–0.06 mm.	spectral region 10– 30 A

In the case of chlorophyll *b*, constant values at the red maximum have been obtained for spectral regions isolated up to 38 A. The slit widths employed in all the absorption measurements were within these ranges.

The radiation which reaches the solution has passed through much crystal quartz and has been reflected many times; it is therefore likely to be partially polarized. Absorption values for chlorophyll *a* at 6600 and 4290 A were unchanged when a Polaroid disk was placed in the light path between the source and the condensing lens or directly before the absorption cell. Observations were made with the Polaroid disk at several rotational positions perpendicular to the light beam.

Determinations made on chlorophyll *a* with the following lines from a low pressure mercury arc agreed with those made on the same solution at the same wave lengths with a Mazda source: 4047, 4078, 4358, 4916, and 5461 A.

Absorption values of chlorophyll *a* solutions at the 4290 A maximum were observed when the following glass filters were placed in front of slit 1 of the monochromator: Jena BG7 (absorbs from 6400 A to the red limit of photocell), Corning 385 (absorbs below 3700 A), Corning 585 (absorbs from 5000 to 6900 A), BG7 + 585, and BG7 + 585 + 385. At the 4720 A minimum, Jena VG9 (absorbs from 6400 A to the red limit and from 4400 A to the ultraviolet limit of Mazda source), Corning 511 (absorbs from 5000 A to the red limit), and both in series were used. No change was observed in the absorption coefficient in any of these experiments, demonstrating that error due to scattered radiation from the Mazda source is negligible under the conditions of these measurements.

The blue and red maxima of chlorophyll *a* preparations are found consistently at 4290 and 6600 A and those of chlorophyll *b* at 4530 and 6425 A, respectively. Determinations in these regions were made at 10-A intervals. Other maxima and the minima are broad in comparison.

A straight line relationship between $\log_{10} \frac{I_0}{I}$ value and concentration has been found at 4720, 5050, 4290, and 6600 A for chlorophyll *a* in ether, for the concentration range employed.

Comparison of data

The curves in general are higher at the blue and red maxima than are those of other workers and lower at the minimum in the blue-green region (note particularly wave lengths 4720 and 5050 A for *a* and 4980 and 5200 A for *b*) as shown in figures 3 and 4. The values are equally accurate at the minima and maxima. The 4720 A minimum of *a* is particularly sensitive to the presence of carotenoid impurities. One % beta carotene would raise the absorption coefficient from 0.93 to approximately 3, an increase of 200%. This may explain high values at this wave length (see samples 1 and 6, table 1). Values at 4290 and 5050 A are much less sensitive to such impurities. Values at 4290, 5050, and 6600 A are sensitive to small amounts of pheophytin. It is apparent from figure 3 that most values from other laboratories at these wave lengths fall between our curves for component *a* and the corresponding pheophytin. The same is true for *b* at analogous wave lengths.

The curves show significant disagreement with those of Mackinney (**7**) and Zscheile (**13**). Both workers employed the spectro-photoelectric method of measurement. Only maxima and minima and certain other pertinent points from Zscheile's curves are plotted for comparison in figures 3 and 4. The conditions of preparation and measurement which are reported here are considerably improved over those under which Zscheile worked (**13**). Only two concentrations were employed in these 1934 measurements, and some $\log_{10} \frac{I_0}{I}$ values, particularly at the lower minima, were therefore not in the optimum range.

Inspection of the points plotted in figure 3 from Zscheile (**13**) indicates that contamination with neither pheophytin nor colorless impurity alone could account for all the differences (see wave lengths 5050, 5275, and 6300 A). High pheophytin content is not indicated, but colorless impurities were probably present because the adsorbent was removed merely by filtration through filter paper. It is interesting to note that Zscheile's values at 4290 and 6600 A are both 75% of those reported here. Some values, such as those at 5050, 6600, and 4290 A, might be considered to result from a combination of these two sources of error. R_a values as high as that of Zscheile have not been obtained with the two brands of talc available here, when solutions were dried. These and other data indicate that samples of talc differ considerably in the amount of pigment decomposition resulting when chlorophyll is adsorbed on them. The R_a values differ by a factor as high as 3 when sucrose and talc adsorbents are compared (dried preparations).

Similar considerations apply in the case of *b;* however, larger differences are involved at certain wave lengths. In 1936 Zscheile (**14**) prepared component *b*

by extending his earlier method (**13**) to include two successive adsorptions on sucrose. The last traces of *a* were observed as a thin layer separated from the *b* layer on the last column. Sucrose was removed by washing with water and evaporating the solution to dryness. Maximum specific absorption coefficients of 147 and 49.5 were obtained at the blue and red maxima, respectively. These values approach those reported by MACKINNEY (**7**). His reported values at the blue and red maxima for both components are somewhat lower than ours, and his values at the low minima are higher. However, he does not claim great accuracy for these latter values.

The curves of chlorophyll from Sandoz, Ltd., indicate the magnitude of error that may arise from the use of such preparations as spectroscopic and colorimetric standards. These samples, in common with many others, show high absorption in the region near 5000 A. The present data indicate why MEYER (**8**) obtained increased values near 5300 A after drying his "native chlorophyll" and allowing it to stand. Drying rendered his standard chlorophyll unreliable. We found that when a chlorophyll *a* solution in ether was dried by nitrogen which was purified by passage over hot copper, the value of R_a decreased as much as when dried in air. MEYER also found that increase of values near 5300 A was independent of the presence of oxygen.

HAGENBACH *et al.* (**2**) used chlorophyll from Sandoz, Ltd., and—after repurification by adsorption—measured the spectra with König-Martens and Hilger spectro-photometers. Their value of 231 for chlorophyll *b* at 4499 A is too high to plot in figure 4. They obtained equal values for *a* and *b* at the red maxima. We offer no explanation for the unusually high values of these workers.

RUDOLPH (**9**) prepared *a* and *b* by the methods of WILLSTÄTTER and STOLL and of SCHERTZ and observed the spectra with a König-Martens spectro-photometer. His curves do not cross in the green and red regions. They are so low and lacking in detail as to indicate gross impurity, degradation, or both.

HEIERLE (**3**) employed chlorophyll supplied by STOLL and used a Zeiss grating spectrograph with a Baly absorption cell. His values are in general low in the red and blue regions and high in the green. His curve for *b* is particularly poor in the red region, since the shelf near 6600 A indicates contamination with either component *a* or a pheophytin.

We have observed that the initial degradation by drying of chlorophyll solutions causes the absorption curve to fall intermediate between the curves for the pure component and its corresponding pheophytin, at most wave lengths. Very large amounts of degradation, as caused by exposure to light at room temperature, cause decomposition of pheophytin also, and thus produce a general flattening of the entire curve, with attendant disappearance of detail. This complex course of

events makes impossible at the present time the accurate treatment of data from various laboratories, at least so far as definite statements of detailed causes for differences are concerned.

MACKINNEY (6) has stressed the importance of obtaining the chlorophyll components in a pure solid form. On the basis of results presented here, it does not seem probable that the components of chlorophyll, in a stable and pure form, will be generally available for use as spectroscopic standards.

It is hoped that careful observations of certain precautions discussed here may contribute to an early agreement among workers in this field of pigment research.

Summary

1. An improved method for preparation of chlorophyll component solutions with reproducible spectroscopic properties is presented. The method is rapid and simple; sucrose is used, both for filtration of precipitated pigment and for separation of components by adsorption.

2. The absorption spectrum in ether solution has been shown to be very sensitive to previous treatment of the solution. It is emphasized that drying must be avoided and that spectroscopic observations must be made very soon after purification.

3. The spectra of acidified chlorophyll solutions are presented, and their relation to ordinary degradation effects is discussed. Some comparisons with reported values are made.

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OÖGENESIS AND FERTILIZATION IN PINUS LAMBERTIANA AND P. MONOPHYLLA

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(WITH TWENTY-SIX FIGURES)

Introduction

An investigation of the cytological features associated with the maturation and fertilization of the egg was undertaken in two species of pines occurring in southern California. Both produce large ovules that may be easily removed from their cones and dissected. The cones of *Pinus lambertiana* reach a greater length than those of any other species, but being borne on tall trees, are difficult to obtain. Although much smaller, the cones of *P. monophylla* occur in great abundance on trees of low stature having branches close to the ground.

Material and methods

Nearly all the material of *Pinus lambertiana* was collected in the San Bernardino Mountains north of Redlands, California, at elevations ranging from 5700 to 7200 feet. Collections were made daily from June 21 to June 24, 1935. As might be expected, cones from a lower altitude were in a later stage of development than those from a higher altitude. On June 21, material gathered at 5700 feet showed many cases of fertilization, while at 7200 feet the archegonia revealed an undivided central cell, or occasionally one in process of division. On June 24, fertilization stages were found only in material obtained at 6400 feet, that collected at lower altitudes being too old and at higher altitudes too young. At any given elevation, about 5 days were found to elapse between the cutting off of the ventral canal cell and fertilization.

Material of *Pinus monophylla* was obtained in Cuddy Canyon, 2–4 miles west of the point where Los Angeles, Kern, and Ventura counties meet. It came from various elevations between 4300 and 5200 feet. Material collected on June 8 at 4300 feet included an abundance of fertilization and young proembryonal stages; that gathered at higher elevations was younger. On June 15, material growing at 4300 feet had reached a proembryonal stage, with walls formed and suspensors elongating; at 5200 feet the ovules were much younger, only a few having reached the fertilization stage. Collections made on June 1, 1936, and May 31, 1937, at 4300 feet showed the nucleus of the central cell just ready to divide in some ovules, in others just having divided. Another collection at the same elevation on June 4, 1937, yielded many cases of fertilization.

In both species, the interval between pollination and fertilization is almost exactly one year. In *P. lambertiana* the staminate cones opened a day or two after ovules in fertilization stages had been collected from the same trees. In *P. monophylla* the staminate cones at the time of fertilization were just beginning to shed their pollen and the young ovulate cones were just emerging from their buds. In the large cones of *P. lambertiana* the ovules near the apex might be in the mature-egg stage, fertilization not yet having occurred, and those near the base in an early proembryonal stage, with free nuclei at the center or base of the egg. In the small cones of *P. monophylla*, on the other hand, the ovules are more nearly in the same stage of development throughout. At any particular time, however, some variation occurs among cones taken from the same tree, and still more among cones taken from different trees growing at the same elevation.

Upon removal from the trees, the cones were wrapped in waxed paper and placed in air-tight containers. Two or 3 hours later they were brought to the laboratory for dissection, the ovules meanwhile having lost none of their turgidity. Because of the large size of the ovules, the female gametophyte could easily be removed, a procedure greatly facilitating penetration of the fixing fluid. Except in early stages, it was usually possible to retain the nucellus in place at the apical end of the female gametophyte.

The material was satisfactorily fixed in Navashin's fluid, prepared according to the formula given by CHAMBERLAIN (4). Shrinkage of the egg, which nearly always follows the use of ordinary methods, was avoided by leaving the material in the fixing fluid for at least 3–4 days, washing thoroughly, and carrying it through a very closely graded series, first of ethyl alcohol and then of xylol. The sections were cut 12 μ thick and were stained in iron-alum haematoxylin.

Historical résumé

The literature dealing with the cytological aspects of fertilization in conifers is not extensive, the principal papers being those of BLACKMAN (2) on *Pinus sylvestris*, FERGUSON (6, 7) on *P. strobus*, HUTCHINSON (8) on *Abies balsamea*, and BEAL (1) on *P. banksiana*. Oögenesis was studied by both BLACKMAN and FERGUSON, while CHAMBERLAIN (3) on *P. laricio* was concerned mainly with oögenesis. Fertilization has been described, in considerably less detail and incidental to other features of the life history, by MURRILL (12) in *Tsuga canadensis;* LAWSON (9, 10, 11) in *Sequoia sempervirens, Cryptomeria japonica*, and *Cephalotaxus drupacea;* NORÉN (14) in *Juniperus communis;* and NICHOLS (13) in *J. communis* var. *depressa*.

The most recent study of fertilization in conifers, that of BEAL (1), deals with the behavior of the chromosomes following the conjugation of the male and female nuclei. It confirms in all essential respects and adds details to the earlier investiga-

tions of BLACKMAN, FERGUSON, and others, who found that in the first embryonal mitosis the paternal and maternal chromosomes split longitudinally, as in any other vegetative mitosis, and that the number of chromosomes in the fertilized egg is diploid.

BEAL's results are in sharp disagreement with those of HUTCHINSON (**8**), who reported that the members of the paternal set of chromosomes pair with those of the maternal set, that each chromosome undergoes transverse rather than longitudinal segmentation, and that the segments thus formed separate, half going to each pole of the spindle. After pairing, but before separation, the chromosomes were seen to twist about each other and become looped, a behavior suggestive of that occurring in meiosis. HUTCHINSON reported that the number of pairs of chromosomes is haploid; BEAL found no pairing and no transverse segmentation, but a diploid complement of chromosomes, each of which is split longitudinally.

Investigation

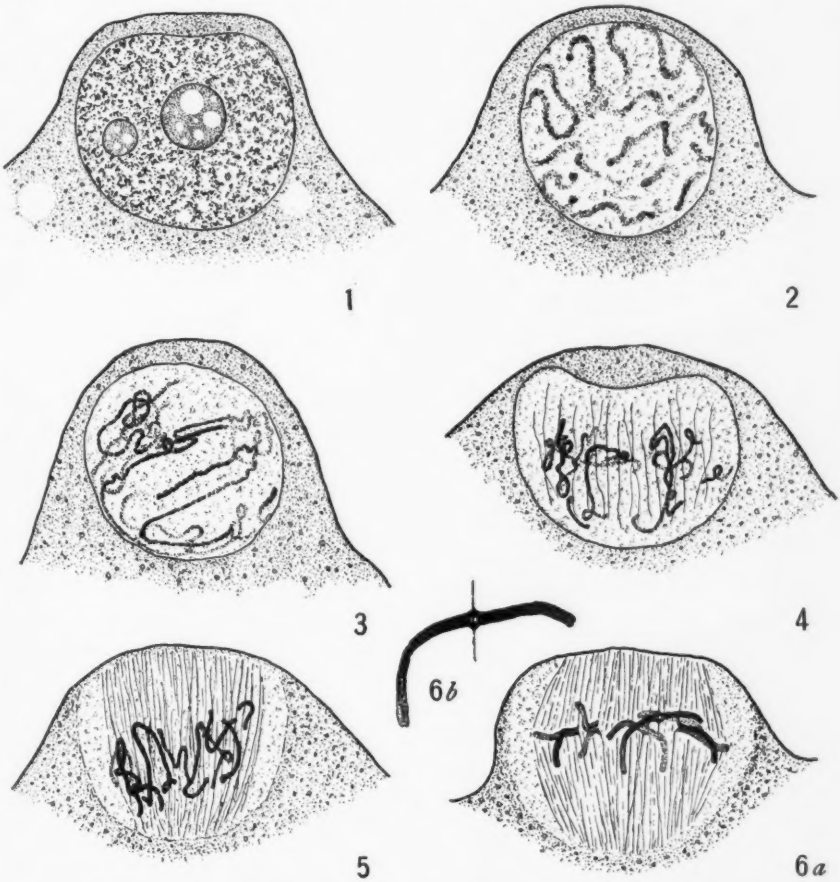
Except where otherwise noted, the following statements apply to both species of *Pinus* studied.

CENTRAL CELL

The cytoplasm of the central cell consists of a finely granular ground substance, without fibrous elements, containing numerous vacuoles confined chiefly to the central region and ranging in diameter from 5 to 100 μ (occasionally up to 200 μ). Deeply staining food particles are distributed in great abundance throughout the cytoplasm. At first they are very small and seem to form an irregular reticulum, but later increase in diameter up to 2 μ and appear separate and distinct. The "proteid vacuoles" of earlier writers are organized within the central cell as it matures. They consist of a distinct membrane inclosing food particles that have the same appearance as those occurring in the general cytoplasm. They attain a diameter of 30–40 μ. At first these proteid bodies are restricted to the bottom and sides of the cell, where the cytoplasm is less vacuolated, but as the vacuoles in the central region decrease in size and number, they become more abundant and more evenly distributed.

The nucleus is situated at the extreme apical end of the central cell, just below the single tier of neck cells (fig. 1). It is most commonly ellipsoidal and flattened or concave above, varying in its greatest diameter from 45 to 55 μ. A small amount of cytoplasm, sometimes containing several small vacuoles, is present above the nucleus. The nuclear material is more coarsely granular than the cytoplasm, but stains almost as lightly. Apparently it consists of a single substance forming an irregular network in which no linin can be distinguished. The nucleus generally contains several nucleoli, the largest of which may be 15 μ in diameter. Commonly two nucleoli are present, one being about twice as large as the other.

They stain somewhat darker than the nuclear network and are conspicuously vacuolated. FERGUSON (**6, 7**) states that the largest nucleolus is invariably central in position, but this is not true of either of the present species.



FIGS. 1–6.—Early stages in division of central cell nucleus of *P. monophylla:* fig. 1, resting nucleus; fig. 2, early stage in differentiation of chromatic threads; fig. 3, later stage, chromatin confined to periphery of nucleus; fig. 4, chromosomes and spindle fibers; fig. 5, disappearance of nuclear membrane; fig. 6*a*, equatorial-plate stage; fig. 6*b*, single chromosome showing longitudinal splitting and fiber-attachment points. Fig. 6*b*, ×1250; others, ×625. Figs. 3, 5, and 6 represent one section from a series of three; figs. 2 and 4, one section from a series of four.

Some preparations show a slight withdrawal of the central cell from the neck cells, while others do not. Often torn strands of cytoplasm extend into the in-

tervening space, indicating that the withdrawal is due to shrinkage arising from imperfect methods of preparation. For the same reason the central cell occasionally is pulled away from the archegonial jacket. By the time the division of its nucleus takes place, the central cell has reached an average diameter and length of approximately 400 and 700 μ, respectively, but sometimes a length of 1000 μ may be attained, especially in *P. monophylla*. As a rule the vacuoles have disappeared from the cytoplasm, but often a few persist in the apical region. FERGUSON (**6, 7**) finds only a few proteid bodies at this stage, but in my material they are abundant even somewhat earlier.

The number of archegonia in a single ovule is somewhat variable, but is most commonly five in *P. lambertiana* and generally three in *P. monophylla*.

DIVISION OF CENTRAL CELL

As previously stated, the resting nucleus seems to contain a single granular material, somewhat coarser than the cytoplasm, and forming an interrupted reticulum. Preparatory to nuclear division, some of this material becomes aggregated to form loose clumps that show no regularity in form or distribution. By a further consolidation of their particles, these irregular clumps soon attain a more definite shape, and their appearance now suggests that they represent differentiated strands of chromatin (fig. 2). As it loses its granular character and becomes more compact, the chromatin assumes a deeper stain. Meanwhile the nucleoli disappear. Soon the chromatin becomes drawn out into long slender threads sharply differentiated from the surrounding granular material of the nucleus (fig. 3). They do not appear to form a continuous spireme. These threads have a moniliform appearance and portions tend to be highly convoluted. In some preparations the chromatin at this stage lies in the peripheral portion of the nucleus.

A later stage in the division of the central cell nucleus reveals an increased consolidation of its chromatin to form definite chromosomes and the appearance of spindle fibers (fig. 4). The chromosomes are very long, slender, and coiled. A moniliform structure is no longer apparent. At first the chromosomes are distributed throughout the nucleus, and because many are cut by the microtome knife, it was not possible to determine their number. At this stage the chromosomes are so slender that it could not be ascertained whether they are longitudinally split, although there is some evidence that such is the case. The spindle fibers make their appearance entirely within the nuclear membrane. They are relatively few in number and extend vertically in an irregular manner.

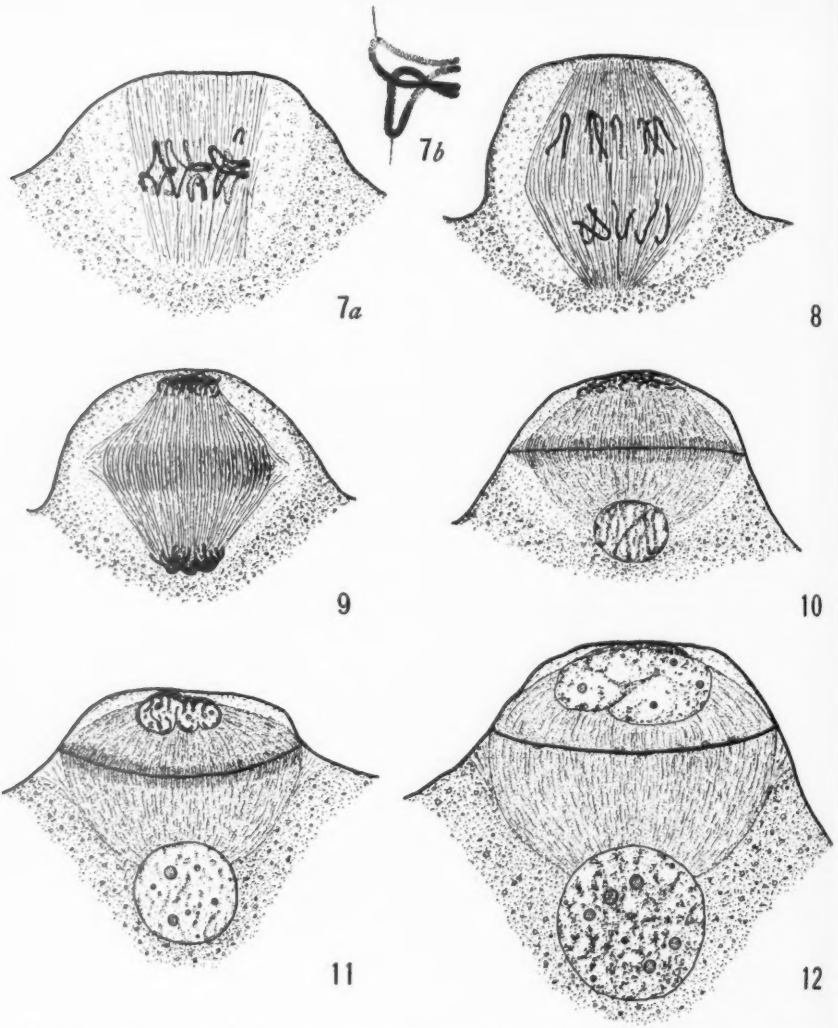
Presently there is formed a well-defined, intranuclear, multipolar, diarch spindle extending throughout the entire length of the nucleus (fig. 5). It is as broad above as at the equator (30–35 μ), or nearly so, but is usually somewhat narrower below. The chromosomes are now grouped in the central portion of the spindle and are

spreading out in a horizontal plane. They are still long, slender, and much curved, the longest being 500–550 μ. Often longitudinal splitting can be clearly seen, and sometimes it is possible to discern fiber-attachment points. Lying outside the spindle and reaching to the nuclear membrane, which is now breaking down, is the lightly staining granular material not previously involved in the formation of chromatin. During this stage and subsequent ones, the nucleus generally lies so close to the apical end of the central cell that little or no cytoplasm is present above it.

Many metaphases were studied. The nuclear membrane has now almost or entirely disappeared. The spindle remains broad at the poles, granular material derived from the resting nucleus surrounding it on all sides. Often the spindle is as broad at each pole as at the equator, but usually it is slightly narrower below. BLACKMAN (**2**) states that the spindle is bluntly pointed at both ends; FERGUSON (**6, 7**) finds that the lower end forms a single pole while the upper remains multipolar. By this time the chromosomes have become much shorter, as well as thicker, denser, and more or less U-shaped (fig. 6*a*). They are arranged somewhat regularly on the equatorial plate, the ends tending to point outward. The fiber-attachment points are now conspicuous, being situated at or near the middle of the chromosomes (fig. 6*b*). In most cases the presence of 12 chromosomes can be determined with certainty. Longitudinal splitting can be more readily observed as the chromosomes continue to shorten and thicken. At the equatorial-plate stage the chromosomes are much twisted, particularly near their ends. This is very apparent somewhat later, when the halves of each split chromosome separate (fig. 7*a*, *b*).

During the anaphase the chromosomes are V-shaped or narrowly U-shaped (fig. 8). In both species, 12 chromosomes going to each pole can be easily counted. Although the nuclear membrane has disappeared, the granular material persisting from the resting stage still surrounds the spindle and usually can be distinguished from the cytoplasm. As the chromosomes approach the poles, the fiber-attachment points are somewhat less evident, but still may be recognized. The spindle is now elliptical in outline and narrower at the poles. The lower group of chromosomes becomes more compact, while the upper one spreads out horizontally.

As soon as the chromosomes have reached the poles, a slight thickening of the mantle fibers across the equator of the spindle is the first indication of cell-plate formation (fig. 9). In a slightly later stage the spindle has broadened at the equator by the appearance of additional fibers, and the cell plate has formed across it (fig. 10). The lower group of chromosomes now forms a compact, spherical, lightly staining mass inclosed by a thin nuclear membrane, while the upper group, which stains more intensely, usually becomes flattened out against the cell wall. By this time the persistent granular material of the resting nucleus is barely discernible,



FIGS. 7–12.—Late stages in division of central cell nucleus of *P. monophylla:* fig. 7*a*, late metaphase; fig. 7*b*, single pair of daughter chromosomes nearly separated; fig. 8, anaphase; fig. 9, early telophase; figs. 10–12, later telophases showing cell wall formation. Fig. 7*b*, ×1250; others, ×625. Fig. 7 represents one section from a series of two; fig. 8, one section from a series of three.

having largely merged with the cytoplasm. The egg nucleus is about 15–20 μ in diameter.

The completion of the outward growth of the cell wall that cuts off the ventral canal cell from the egg is shown in figure 11. This wall has now become thickened and slightly concave upward. The egg nucleus, 25–30 μ in diameter, is spherical or sometimes flattened above. The chromosomes are no longer distinct, but at least a portion of their original substance can be identified as faint, narrow, more or less vertical bands. The coarsely granular material has made its appearance in the nucleus and seems to have arisen from disintegration of the chromosomes, at least in part. Usually a number of small vacuolated nucleoli are present also, but these may be wholly absent. In figures 11 and 12 the ventral canal nucleus is flattened and more or less lobed below but shows much the same structure as the egg nucleus. In many cases, however, the ventral canal nucleus does not reach the resting condition, its chromatin remaining as an indistinct mass that soon disorganizes without forming a nuclear membrane. The ventral canal cell persists as a small, lenticular, deeply staining mass lying between the egg and the neck cells.

In *P. sylvestris*, BLACKMAN (2) describes the nucleus of the ventral canal cell and that of the egg as exactly similar, stating that the former "surrounds itself with a membrane and goes through the earlier stages, at least, of the normal process of return to the resting condition . . . . but soon after its formation loses its membrane and appears as a number of irregular rods and clumps." FERGUSON (6, 7), on the contrary, finds that the nucleus of the ventral canal cell, although highly variable, rarely approximates the egg nucleus in size and form, and that there are probably instances in which no nuclear membrane appears. It undergoes early disintegration, hardly ever presenting a normal appearance.

MATURE EGG

As the egg nucleus migrates to a central position it becomes ellipsoidal and attains an average diameter and length of approximately 150 and 200 μ, respectively. While it is still under 100 μ in diameter, usually some evidence of chromosome structure may be faintly seen, but this soon disappears. When its maximum size has been reached, the egg nucleus consists of a coarsely granular substance forming an interrupted reticulum and showing no evidence of linin. It exhibits a structure very similar to that of the resting nucleus of the central cell. Sometimes no nucleoli can be recognized, but generally a variable number are present. As a rule there are several small nucleoli, each 5–8 μ in diameter and with a central vacuole. In addition there is usually a single large nucleolus, 20–30 μ in diameter, which contains so many vacuoles that it presents a frothy appearance. Sometimes a great many small nucleoli are seen, in one instance in *P. lambertiana* as many as 130

having been counted in the whole nucleus. The nucleoli are in groups, but the groups are not confined to any particular part of the nucleus.

BLACKMAN (2) considers that the young egg nucleus "consists of a small number of chromatin bands and a few round nucleoli." As it enlarges and moves downward, it becomes filled with a peculiar substance, absorbed from the cytoplasm, that earlier had been designated as metaplasm by STRASBURGER (15), who claimed that it entirely obscures the chromatin. BLACKMAN found that at first the metaplasm is granular and clearly distinct from the chromatin, but later the two are indistinguishable, the whole female nucleus being filled with a reticulum that, although not chromatin, "stains uniformly in a chromatin-like manner." In addition, there is present either a large hollow nucleolus or a number of small nucleoli.

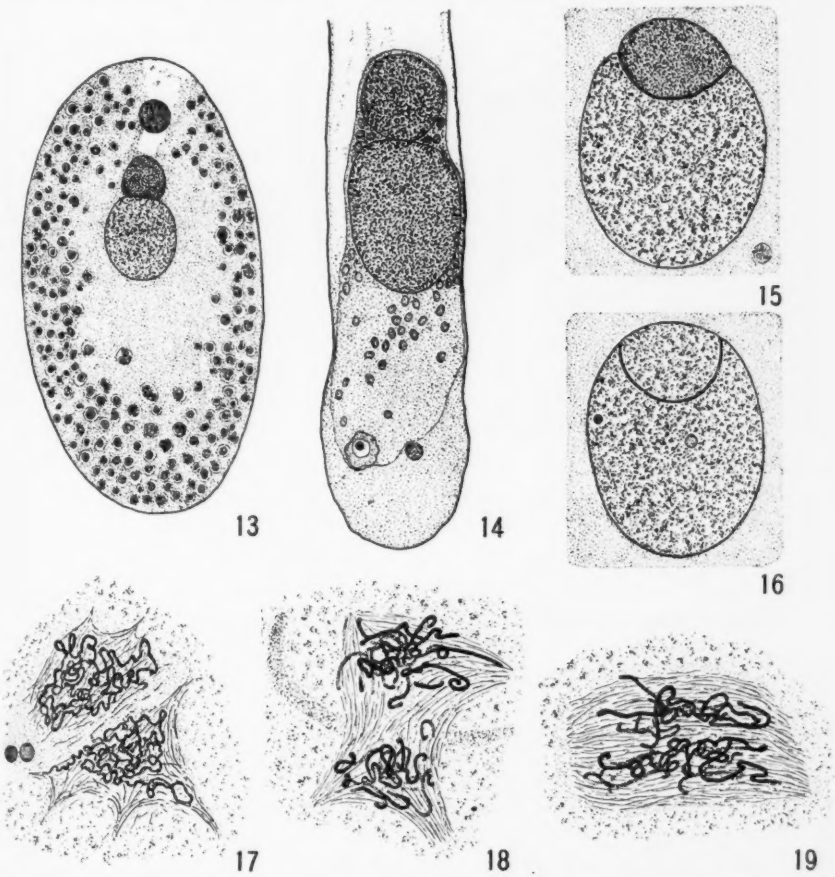
CHAMBERLAIN (3) states that, in the development of the egg nucleus the chromatin takes the form of numerous small nucleoli arranged upon a linin network. This resolves itself into a granular substance, leaving the nucleoli irregularly scattered. He finds that some of the nucleoli contribute to the granular substance, while others come together and give rise to elongated chromatin masses, at which time a nuclear reticulum again appears. CHAMBERLAIN states that "the term metaplasm is not used in the present paper because the network is regarded merely as a somewhat peculiar linin network."

FERGUSON (6, 7) likewise does not recognize the presence of a metaplasmic substance in the egg nucleus. She describes an open interrupted reticulum on which are arranged irregular granules. The linin, always abundant, may form heavy hyaline cords or less conspicuous strands. She finds chromatin to "exist either in the form of irregular granules of varying sizes or apparently dissolved in the linin," but did not observe such a resolving of the chromatin into nucleoli as described by CHAMBERLAIN. FERGUSON reports the occasional occurrence of nucleoli in *P. austriaca* during early developmental stages, but finds them rarely present in *P. strobus* at this time. Later she says, "small nucleolus-like bodies, containing a single vacuole, appear in connection with the nuclear net; and at the same time a slightly larger nucleolus is observed in the lower part of the nucleus, usually in connection with its membrane. As the nucleus continues to grow, this nucleolus also increases in size, gradually becoming large and very vacuolate." FERGUSON finds that there may be several smaller nucleoli, a great many, or none.

After the cutting off of the ventral canal cell, the egg does not increase in size. Its cytoplasm becomes filled with the deeply staining food granules that made their appearance earlier, while the vacuoles are nearly or entirely gone (fig. 13). FERGUSON has expressed the opinion that proteid bodies arise within the nucleoli of the egg and sheath cells. The present study has afforded no evidence that this is true.

Pollen tube

As the pollen tube approaches the archegonia, its contents are massed at the lower end (fig. 14). Here may be distinguished, surrounded by dense cytoplasm containing numerous starch grains, the small tube nucleus and stalk cell, as well as



Figs. 13–19.—Conjugation of sexual nuclei and stages immediately following: fig. 13, two male nuclei within egg, the larger, anterior one in contact with female nucleus, ×95; fig. 14, lower end of pollen tube just before penetration of egg, showing small tube nucleus, stalk cell, and two large male nuclei, ×250; figs. 15, 16, penetration into female nucleus, ×185; fig. 17, male and female chromatin in separate groups, each surrounded by spindle fibers, ×625; fig. 18, later stage, with two groups of chromosomes on common polyarch spindle, ×625; fig. 19, still later stage, with chromosomes still in two groups but spindle becoming diarch, ×625. Figs. 13, 14, and 17 are of *P. lambertiana;* figs. 15, 18, and 19 are of *P. monophylla.*

the two large male nuclei of unequal size. The tube nucleus contains many deeply staining chromatin granules. The male nuclei are inclosed by a common mass of cytoplasm derived from the body cell, which is separated from the general cytoplasm of the pollen tube by a delicate membrane. Each of the male nuclei consists entirely of the same kind of coarsely granular material as that which fills the egg nucleus, but neither at this time nor subsequently are any nucleoli to be seen. The male nuclei lie in contact with each other, the smaller one being behind. In figure 14 the smaller male nucleus measures 50 μ in diameter, the larger one 70×90 μ. FERGUSON (**5, 7**), studying several species of pines, finds the contents of the pollen tube essentially the same as just described, except that the male nuclei, having a dense reticulum, contain no special metaplasmic substance.

CONJUGATION OF SEXUAL NUCLEI

Before the contents of the pollen tube enter the archegonium, a large saclike cavity appears in the upper portion of the egg and persists during its subsequent development, a feature noted by earlier workers. Most of the male cytoplasm enters the egg but does not surround the anterior, functional, male nucleus as it approaches the female. The second male nucleus, and generally the tube nucleus aslo, can be identified in the upper part of the egg, but as a rule the stalk cell does not persist.

When the functional male nucleus reaches the egg nucleus, the two become flattened along the contact surface, while a thin layer of cytoplasm lies between them (fig. 13). As previously pointed out by BLACKMAN (**2**) and FERGUSON (**6, 7**), the male nucleus sinks into the female nucleus, forming an invagination, the nuclear membranes meanwhile remaining intact (fig. 15). This continues until the outer surface of the male nucleus reaches the same level as that of the female (fig. 16). Occasionally a few small masses of cytoplasm are caught between the two nuclei where their membranes are in contact. Generally at this time the egg contains no nucleoli, but sometimes several small ones may be present.

No change takes place in the structure of the functional male nucleus as it approaches the egg nucleus, and when the two first come into contact the former is always denser than the latter. As the male nucleus sinks into the female nucleus it becomes less dense, until finally the two are of equal density (figs. 15, 16). The two male nuclei do not increase in size as they enter the egg, nor do they enlarge subsequently. They are usually ellipsoidal or ovoid, but either or both may be spherical.

FIRST EMBRYONAL MITOSIS

As the portions of the membranes of the fusing nuclei that are in contact slowly break down, two groups of chromosomes are differentiated in the immediate vicinity, one from the male nuclear material and the other from the female. In

each group the chromatin takes the form of attenuated, very slender, convoluted strands; at the same time two groups of spindle fibers make their appearance (fig. 17). Although somewhat irregularly arranged, the fibers in each group tend to form a multipolar polyarch spindle. These spindles, like the male and female chromatin groups, lie close together, but when first apparent are separate. They soon converge to form a common spindle, upon which the two groups of chromatin lie (fig. 18).

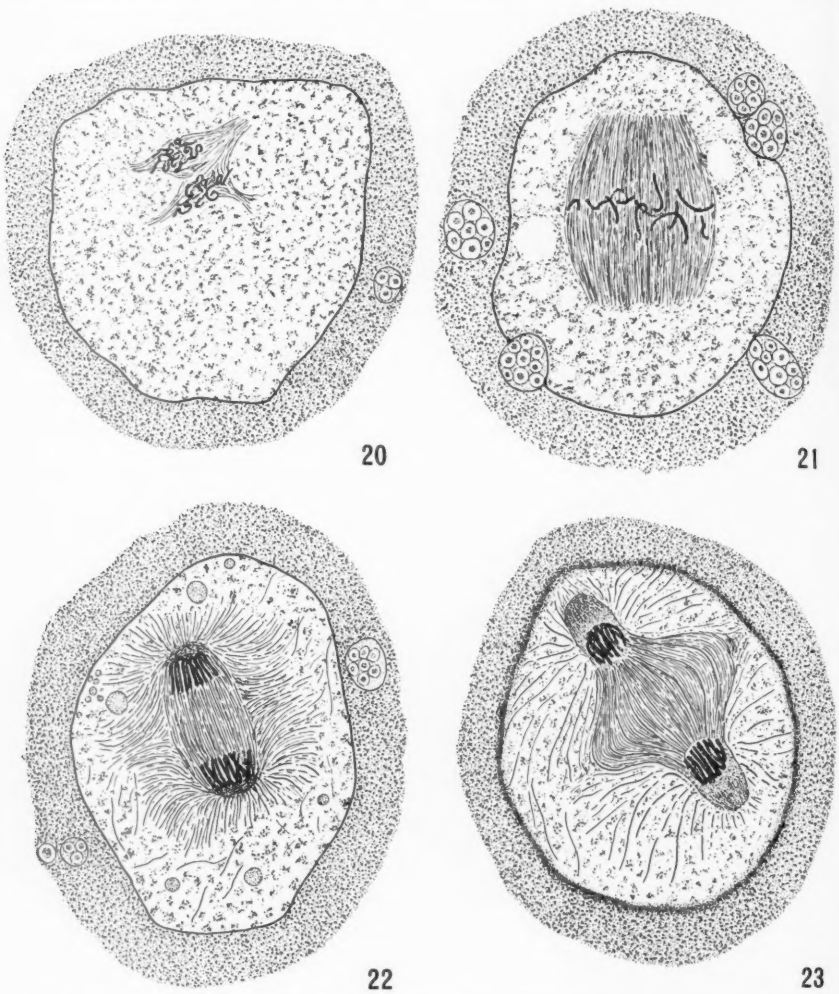
The slender chromatin threads become shorter and thicker, assuming the form of chromosomes. These are long, slender, and curved, sooner or later losing the moniliform appearance characteristic of earlier stages. As the two groups of chromosomes come together, the common multipolar spindle on which they lie gradually becomes diarch (fig. 19). Even now the paternal and maternal groups are recognizable, but soon lose their identity. The female chromatin occupies a relatively small part of the egg nucleus, lying close to the chromatin contributed by the male nucleus. The rest of the egg nucleus contains the same kind of coarsely granular material found before the chromatin became differentiated (fig. 20).

FERGUSON (**6, 7**) finds in each of the two sexual nuclei, after they have come into contact, that the chromatin condenses to form a spireme "studded with irregular granules." This soon becomes coiled and irregularly moniliform, leaving the remainder of the nucleus filled with a granular reticulum composed of linin. Delicate threads appear near the spiremes, later forming an irregular, multipolar, polyarch spindle that often consists of two parts. By this time the chromatic bands have become homogeneous. Finally the spindle becomes multipolar and diarch, "and the achromatic substance not used in spindle-formation has been gradually resolved . . . . into a granular, or finely reticulated structure, which later merges into the general cytoplasm of the egg."

After the paternal and maternal chromosomes have become arranged at the equator of the spindle, they are indistinguishable from each other (fig. 21). The spindle itself, which remains multipolar and diarch, is very broad. It is usually 50–70 μ in diameter, but sometimes may reach a width (in *P. monophylla*) of 120 μ, being then several times broader than long. At first the chromosomes are attenuate and very slender, often showing clearly the moniliform structure characteristic of earlier stages. Soon they become shorter, thicker, and somewhat U-shaped (fig. 24*a–f*). The fiber-attachment points, which are median, are always clearly discernible. Longitudinal splitting of the chromosomes is not evident at first, but soon becomes so, particularly where the spindle fibers are attached.

When longitudinal sections 12 μ in thickness are cut through the mitotic figure, the chromosomes are distributed over a series of at least five or six sections, with the result that many of them are cut into several pieces. But, as BEAL (1) has shown, it is possible to estimate the number of whole chromosomes present in the

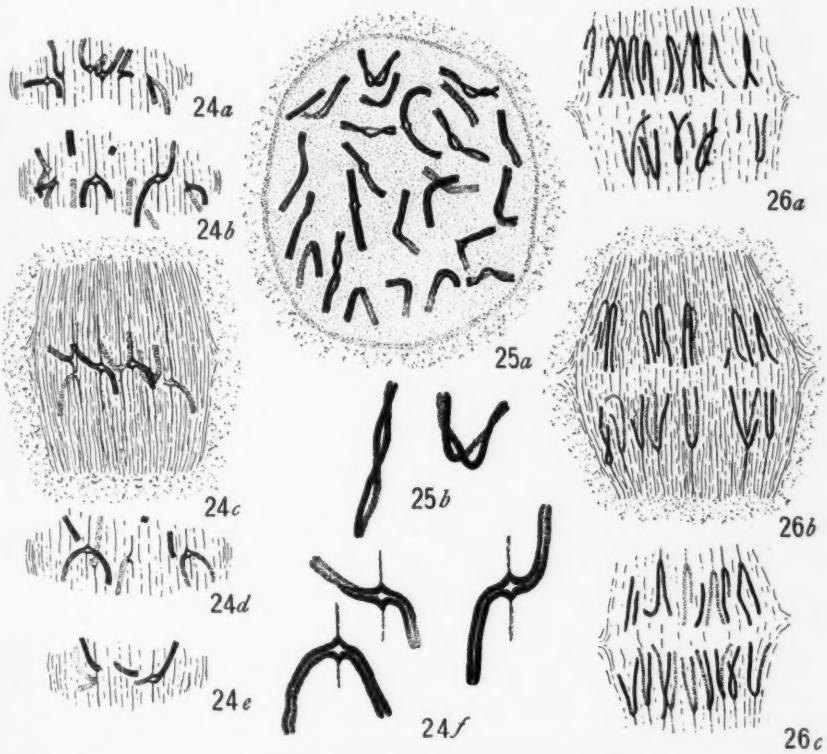
figure before it was sectioned by counting the pieces with fiber-attachment points. Using this method, BEAL found in *P. banksiana* approximately 24 chromosomes at



FIGS. 20–23.—First embryonal mitosis in *P. monophylla*, ×360: fig. 20, egg nucleus with male and female chromatin differentiated; fig. 21, metaphase; fig. 22, anaphase; fig. 23, telophase. Fig. 21 represents one section from series of six; figs. 22 and 23, one section from series of two.

the metaphase of the first embryonal mitosis. In the many preparations of this stage available for the present investigation, in both species, the presence of 24

chromosomes could often be established with considerable accuracy (fig. 24*a–e*). In no case was there evidence of a haploid number of paired chromosomes, as described by HUTCHINSON (8) in *Abies balsamea*.



FIGS. 24–26.—Chromosome behavior during later stages of first embryonal mitosis: fig. 24*a–e*, consecutive longitudinal sections through entire mitotic figure of *P. lambertiana* at metaphase, showing diploid complement of 24 chromosomes, the number in each section with fiber-attachment points being 6, 5, 7, 4, 2; fig. 24*f*, 3 chromosomes from same, showing longitudinal splitting; fig. 25*a*, transection through mitotic figure of *P. monophylla* in late metaphase, showing polar view of diploid complement of 24 chromosomes; fig. 25*b*, two pairs of daughter chromosomes from same, showing twisting; fig. 26*a–c*, consecutive sections through entire mitotic figure of *P. lambertiana* at anaphase, showing 24 daughter chromosomes passing to each pole. Figs. 24*f* and 25*b*, ×1250; others, ×625.

FERGUSON (**6, 7**) states that the two groups of chromosomes remain distinct until oriented on the equatorial plate. At this stage she demonstrated in *P. strobus* the presence of 24 chromosomes in a single cross section. She first observed longitudinal splitting at the metaphase, stating that "each chromatic element divides at the point where the spindle-fibers are attached, forming a small diamond-

shaped opening." BEAL did not find a polar view of the equatorial-plate stage, but of several seen in the course of the present investigation, that of *P. monophylla* (fig. 25*a*) includes the full diploid complement of 24 chromosomes. Each chromosome is longitudinally split and the segments show a pronounced tendency to twist about each other (fig. 25*b*). This twisting becomes apparent in late metaphase stages, both in the first embryonal mitosis and in the mitosis concerned with the cutting off of the ventral canal cell.

Because of the median position of the fiber-attachment points, the daughter chromosomes—upon separation—become V-shaped or narrowly U-shaped. It is not difficult to determine, during the anaphase, that 24 chromosomes pass to each pole (fig. 26*a–c*). At this time the fibers on the outside of the spindle tend to pass outward into the nucleus without extending directly from pole to pole, and this tendency becomes accentuated during the telophase, when a display of achromatic structures is very evident (figs. 22, 23). The telophase is also characterized by a marked broadening of the spindle and the appearance of achromatic material in the form of polar caps. At this time the nuclear membrane of the egg breaks down, allowing the granular metaplasmic material of the nucleus to merge with the surrounding cytoplasm (fig. 23).

As observed by BLACKMAN (**2**), the daughter nuclei resulting from the first embryonal division are at first very small, but later take up metaplasmic substance and often reach one-third the size of the egg nucleus.

SECOND EMBRYONAL MITOSIS

In the second embryonal division BEAL (**1**) states that the chromosomes have approximately the same general form and size as in corresponding stages of the first division; moreover, "the manner of separation at the fiber-attachment points and the indications of longitudinal splitting are practically identical in the two divisions." The present study fully confirms these statements.

Discussion

The presence in the mature egg nucleus of a metaplasmic substance that is absorbed from the cytoplasm and entirely obscures the chromatin, as claimed by STRASBURGER (**15**) and BLACKMAN (**2**), is confirmed by the present investigation. The same substance is present in the male nuclei and in the nucleus of the central cell. No evidence was found supporting the contention of CHAMBERLAIN (**3**) and FERGUSON (**6, 7**) that the nuclear reticulum consists primarily of linin.

The behavior of the chromosomes in the first embryonal mitosis closely parallels that in the division of the central cell nucleus, except that the diploid rather than the haploid number is involved. In both cases the chromosomes arise from

the resting nucleus in the same way, undergo longitudinal splitting, and exhibit conspicuous median fiber-attachment points. In both cases the daughter chromosomes, before separation, twist about each other.

The present study, like those of FERGUSON (**6, 7**) and BEAL (**1**), has demonstrated that the diploid number of chromosomes, each longitudinally split, is present at the metaphase of the first embryonal mitosis. The occurrence, as reported by HUTCHINSON (**8**), of a haploid number of paired chromosomes, each pair consisting of a single paternal and maternal member, was not confirmed. Furthermore, no evidence was seen of a transverse segmentation of chromosomes and of the passage to the poles of the segments as straight pieces. Instead, the segments separating during the anaphase, always in the form of V's or U's, are daughter chromosomes arising from a previous longitudinal splitting.

Summary

1. In both species of pines studied, the interval between pollination and fertilization is almost exactly one year.

2. The resting nucleus of the central cell contains several nucleoli and an apparently homogeneous, coarsely granular, metaplasmic substance forming an interrupted reticulum.

3. During mitosis the chromatin arises from a portion of this substance, appearing as irregular clumps that soon give rise to deeply staining, convoluted threads with a moniliform structure. These form the chromosomes, which gradually shorten and thicken. An intranuclear, multipolar, diarch spindle arises, surrounded by metaplasm not previously involved in the formation of chromosomes or the spindle itself.

4. At the equatorial-plate stage the chromosomes, 12 in number, are split longitudinally and show conspicuous median fiber-attachment points. The daughter chromosomes twist about each other, particularly when they begin to separate. They are V-shaped or narrowly U-shaped as they move toward the poles.

5. The ventral canal nucleus may or may not disorganize before reaching the resting condition. As the egg nucleus enlarges, the metaplasm reappears, soon completely obscuring the chromatin. It then exhibits nearly the same structure as the resting nucleus of the central cell. A variable number of nucleoli are present.

6. The male nucleus consists of the same kind of metaplasmic substance as the egg nucleus, but contains no nucleoli. It sinks into the egg nucleus, the chromatin differentiating in each of the conjugating nuclei after the common membrane separating them breaks down.

7. The male and female chromatin, each surrounded by its own group of spindle fibers, consists of attenuated, very slender, convoluted strands. A com-

mon multipolar, polyarch spindle is formed; this becomes diarch as the chromosomes, arising in two groups, come together.

8. The paternal and maternal chromosomes cannot be distinguished from each other when the equatorial-plate stage has been reached. As they shorten and thicken, they display prominent median fiber-attachment points and conspicuous longitudinal splitting.

9. During the metaphase of the first embryonal mitosis, 24 chromosomes are present, half derived from the male nucleus and half from the female. The longitudinal segments of each split chromosome tend to twist about each other.

10. During the anaphase, 24 V-shaped or narrowly U-shaped daughter chromosomes pass to each pole, as in any other somatic mitosis.

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GROWTH AND DEVELOPMENT IN RANGE GRASSES. I. EARLY DEVELOPMENT OF BOUTELOUA CURTIPENDULA IN RELATION TO WATER SUPPLY

CHARLES E. OLMSTED

(WITH ONE FIGURE)

Introduction

Few published data on developmental histories of range plants are available. Likewise, relatively few critical investigations of their growth and developmental responses to various ecological factors, aside from drought and grazing, have been conducted, under either natural or partially controlled environments. The accumulation and eventual systemization of such data, at least for dominant species or their ecotypes, would seem to be a necessary prelude to any further refinement in the causal interpretation of: (*a*) distributional data for various vegetation types; (*b*) the roles played by the same or different species in different parts of the range; and (*c*) their responses to grazing and drought.

The genus *Bouteloua*, by reason of its size, economic importance, and the differing distributional patterns and roles of its species, has been chosen for investigation. Side-oats grama, *B. curtipendula*, has recently attracted attention because of its apparent drought-resistance and increase in importance in various parts of the mixed and true prairie regions (**12, 13**). It has a wide range, and is thought by some "to be the most promising grama for domestication" (**3**). The present study traces the development of seedlings of this species in relation to water supply under a relatively controlled environment (in the greenhouse of the department of botany, University of Arizona) from November 21, 1939, to March 19, 1940. Concurrent studies on drought response and recovery by seedlings of different ages will be published in the near future.

Methods and environmental conditions

Uniform caryopses, threshed from spikelets collected on the Santa Rita Range Reserve in 1938, were planted $\frac{1}{4}$–$\frac{1}{2}$ inch deep at the rate of twenty-five per pot in the air-dry soil contained in ninety-eight 2-gallon crocks. Each crock, 9 inches deep and 8 inches in diameter, and provided with a center drain, had previously been placed in position on a greenhouse bench and filled with air-dry Tumacacori coarse sandy loam soil, which had been thoroughly mixed and passed through a $\frac{1}{2}$-inch mesh screen. This soil, collected to a depth of 9 inches, at an altitude of 4250 feet on the Santa Rita Range Reserve, had originally supported a dense

mixed stand of perennial and annual grasses. These included *Bouteloua curtipendula*, *B. hirsuta*, *B. filiformis*, *B. aristidoides*, *Aristida divaricata*, *A. adscencionis*, and *Heteropogon contortus*. The soil is dark brown in color, has a high organic content, a pH of 6.5, a moisture equivalent of 6.42 per cent, a permanent wilting percentage of approximately 1.5, and a hygroscopic coefficient of 0.80 per cent. One surface-inch of water (824 cc.), applied to a pot of such soil, raised its average percentage moisture content to 8.25, approximately the field capacity of this soil.

Since it had been shown previously (7) that side-oats grama is sensitive to photoperiod, natural day length was supplemented by Mazda lamps to give a 16-hour light period, in an effort to simulate more closely the conditions under which seedlings of this species normally develop in southern Arizona. No accurate instruments were available for the measurement of daylight intensity. Official records[1] on sunshine and cloudiness show 66 per cent of the days clear and 17 per cent each of cloudy and partly cloudy days during the experiment. The cloudiness was concentrated at intervals, primarily before February 1.

A shaded hygrothermograph, placed among the pots, was operated continuously. Maximum daily air temperatures ranged from 71° to 89° F. until the middle of February, although most fell between 80° and 85° F., except on cloudy days. Maximum temperatures between 90° and 100° F. were recorded for 9 days between February 17 and March 17. Minimum daily temperatures ranged from 60° to 72°, although most were between 65° and 69° F. Relative humidity ranged 80–95 per cent through the 10 to 14-hour period during which the ventilators were closed each night, but fell to a minimum below 40 per cent for several hours in all but 17 days of the experiment.

On November 21, 1939, and again on November 24, water equivalent to 0.2 surface-inch was added to each pot with a bottle sprinkler. The pots meanwhile were covered with light paper to retard surface evaporation until the evening of November 26. Germination was excellent and uniform, and on the morning of November 27, after the application of 0.4 surface-inch of water to each, the pots were divided by randomization into series, three of twenty-six and one of twenty pots, to be watered at intervals of 3, 6, 12, and 20 days, respectively, thereafter. All pots were given 0.4 surface-inch at each watering, those continuing to the end of the experiment thus receiving a total of 15.6, 8.0, 4.4, and 2.8 surface-inches in order of decreasing watering frequency. These series will hereafter be referred to as 3's, 6's, 12's, and 20's. In table 1 is shown the minimum percentage soil moisture content at the close of certain watering intervals in each series, as determined on pots which were taken down on the various dates. The addition of 3.3 per cent to the figures will indicate the maximum average water content for

[1] Obtained by courtesy of Mr. H. V. SMITH.

the whole soil mass in the pots in each series during the following watering interval. Repeated observations and experiments on other pots indicated a more uniform distribution of water added to the drier soil than was expected, on the assumption that penetration downward would occur only as successive layers were wetted to field capacity. The data indicate the increase in aridity which occurred in all series in the later stages of the experiment, owing to the increasing rate of water loss by the developing plants and to the higher rate of evaporation from the soil surface due to increasing temperature and decreasing air humidity.

Because of its significance in adventitious root establishment, daily early morning observations were made on the wetness of the soil surface in each pot, as well as on the evidences of water deficit in the plants. The 3's were wet each morning until February 10, after which the surface soil was dry on the third morning following watering until February 27, and thereafter on the second morning until the close of the experiment. Afternoon wilting appeared in this series about the middle of February on the third day of each watering interval, gradually increasing in severity and duration so that by March 2 and thereafter the plants remained wilted through the night preceding the next watering. The surface soil in the 6's was dry on the third to fifth morning of each interval until February 2, and on the second morning thereafter to the end of the experiment. Severe afternoon wilting, first recorded in this group on January 24, persisted overnight on the sixth day of the interval on February 7. Thereafter the duration of water deficit in each interval increased to a 3-day maximum of continued wilting at the close of the experiment. The 12's showed surface soil dryness by the third morning following watering until January 16, when it was dry on the second. The latter condition continued to the close of the experiment. Wilting of overnight duration gradually increased from a minimum of 2 days at the close of an interval in the first month to a maximum of 9 days out of 12 at the close. In the 20's the soil surface was dry on the second morning following watering in all intervals but the one beginning on January 6, which required 3 days for this condition to appear, thus allowing the development of the few adventitious roots which were estab-

TABLE 1

MINIMUM SOIL MOISTURE PERCENTAGE, DRY-WEIGHT BASIS, ABOVE HYGROSCOPIC COEFFICIENT, AT CLOSE OF CERTAIN WATERING INTERVALS

SERIES	DEC. 9	DEC. 17	DEC. 21	JAN. 2	JAN. 6	JAN. 14	JAN. 26
3's	6.51	.....	8.42	12.21	.....	7.72	5.73
6's	3.04	.....	3.02	4.03	.....	3.82	0.90
12's	2.18	.....	1.66	1.62	.....	1.67	1.12
20's	.....	1.51	.....	.....	1.51	.....	0.77

lished in this series. Continued wilting in this series increased from 2 days at the close of the first interval to 13 days at the last. In all series the surface 1–1½ inches of soil became air-dry in 24–48 hours after the first record of morning dryness.

FIG. 1.—Upper row (left to right), 66-day old plants of *Bouteloua curtipendula* watered at intervals of 3, 6, 12, and 20 days and supplied total of 8.4, 4.4, 2.4, and 1.6 surface-inches per pot respectively; lower row, same at 118 days, and supplied total of 15.6, 8.0, 4.4, and 2.8 surface-inches.

On December 17 the plants were thinned to ten per pot, selected for uniformity. Variation obviously due to genetic differences was less than might have been expected from the mixed parent population. Variation between pots in any one series was also slight, as shown by repeated examination when pots were removed from each series for various purposes.

On December 9 and 21, January 2, 14, and 26, and March 19, plants from one

pot each of the 3's, 6's, and 12's were carefully washed out of the soil, separated from one another, and allowed to dry out in papers until they could be examined in detail. The same procedure was followed for the 20's on December 17, January 6 and 26, and March 19. Prior to further examination, the plants were soaked in water for 24 hours. Early critical examination showed that identifying characteristics of parts alive or dead when the pots were taken down were well preserved by this drying procedure, which therefore introduces no error into the data. Results presented in the following section are for the most part the averages of the measurements for the ten individuals in each pot taken down on the various dates. Figure 1 shows representative pots from each series on January 26 and March 18.

Data on adventitious root primordia include records on all which could be located by careful dissection under a binocular microscope at 14×, whether or not they had burst through the outer stem tissues or leaf bases. Only those leaves on either primary or secondary axes were counted which showed tips emergent above the surrounding leaves. Axillary buds (tillers) were located where necessary by stripping off their axillant leaves, but were counted only when they exceeded 1 mm. in length.

Results

Primary root system

The primary root system consists only of a taproot and its branches. The primary root elongated rapidly, reaching average lengths of about 9 and 16 cm., in the 6's and 12's, 12 and 18 days after the first watering. Penetration was less in the 3's, but numerous laterals were produced in the upper 2 cm. of soil, in contrast to their absence there in the other series, where the surface soil dried rapidly. Laterals at lower depths averaged about seven per centimeter of primary root in all series, but few laterals of the second order had developed in any 18-day plants. Essentially the same stage of development was shown in the 20's when 26 days old as in the 18-day 6's and 12's, the primary root then averaging 18 cm. long. Further increases in length had occurred in the 30-day 3's, 6's, and 12's to 18 cm. in the first and to 26 cm. in the latter two series. Some 42-day plants in the 3's showed death of the entire primary root system, and it was apparently dead in all plants examined in this series at 66 and 119 days. With adequate establishment of adventitious roots, it probably becomes nonfunctional when 6–10 weeks old, although the actual cause of death in the 3's may have been lack of aeration during the period of excessive soil moisture centering around January 2 (table 1). Unexpected death of adventitious roots also occurred in the 3's at this time. In the 6's, all primary roots examined were alive at 42 days and dead at 66. At 119 days, however, they were still alive on two plants with one or no

established adventitious roots, but were dead on all those with an adequate secondary root system. In most of the 12's and 20's, the primary roots apparently remained alive until the close of the experiment, and in length and degree of branching in individual plants showed a strong inverse correlation with their numbers of functional adventitious roots.

Primary stem and leaves

Seventy-two hours after the initial watering, tips of the coleoptiles were emergent 5–10 mm. above the soil surface. Twenty-four hours later, elongation of the first internode (mesocotyl) had ceased, bringing the coleoptilar node to the ground line, or in a few cases as much as 8 mm. above it. These latter plants, if among

TABLE 2

AVERAGE GROWTH DATA ON THINNED PLANTS, 26 DAYS OLD DECEMBER 17

	Series			
	3's	6's	12's	20's
Total number of plants	152	188	152	98
Surface-inches water supplied	3.2	2.0	1.2	0.8
Number, primary foliage leaves	3.6	3.5	3.2	2.9
Length to tip, cm.	12.0	11.1	9.1	6.0
Dry weight of top, mg.	10.1	7.9	5.5	3.3
Visible adventitious roots	1.3	1.0	0.8	0.4

the 6's, 12's, or 20's, rarely succeeded in establishing adventitious roots. On the fourth or fifth day the first foliage leaf burst through the coleoptile, by the end of the sixth had attained a length of 25–50 mm., and on December 3, 12 days after first watering and 3 days after the beginning of divergence in treatment, was 60–90 mm. long in all series. The second leaf tip also emerged in most plants on December 3.

The effect of variation in water supply on growth and development of tops, beginning to be noticeable on December 9, as shown by data for one pot in each series in table 3, was marked by December 17, the seventeenth day following divergence in treatment, as seen in table 2, where the data are based on all plants removed in thinning. Growth rates in this species in this soil are obviously affected by decreasing soil moisture before the latter has fallen to the level of the permanent wilting percentage, which was not reached in any of the pots in the early stages of the experiment.

The rate of increase in foliage leaf number with age, and of plant height, as measured by distance from the ground to tip of the leaf uppermost when all were

extended, is shown in table 3. Variation in water supply, within the range of this experiment, apparently had less effect on the number of leaves produced by the primary axis than upon plant height. The latter was affected by differences in culm elongation, as well as by differences in leaf size, produced through the dwarfing effect of deficient water supply in the drier series. The number of basal internodes which matured without elongating, and the age at which elongation began, as well as the number and length of expanded segments, were all affected.

TABLE 3

PLANT HEIGHT,* TOTAL NUMBER OF PRIMARY LEAVES, AND OF DEAD OR DYING PRIMARY LEAVES

Series	Date and age in days							
	Dec. 9 (18)	Dec. 17 (26)	Dec. 21 (30)	Jan. 2 (42)	Jan. 6 (46)	Jan. 14 (54)	Jan. 26 (66)	Mar. 19 (119)
3's								
Height, cm.	5.9	.....	14.0	27.9	.....	34.0	53.8	64.6
No. of leaves	3.1	.....	4.1	5.9	.....	7.4	8.6	11.8
Dead or dying leaves	0.0	.....	1.2	4.2	.....	5.6	6.4	10.4
6's								
Height, cm.	7.0	.....	12.4	23.6	.....	35.6	44.4	43.0
No. of leaves	3.0	.....	4.4	5.7	.....	6.7	7.9	10.0
Dead or dying leaves	0.0	.....	0.9	3.3	.....	4.7	6.6	9.5
12's								
Height, cm.	5.4	.....	8.7	23.3	.....	27.5	30.7	34.9
No. of leaves	2.2	.....	4.5	5.7	.....	6.5	7.1	9.1
Dead or dying leaves	0.0	.....	0.3	3.1	.....	4.2	5.4	9.0
20's								
Height, cm.	.....	6.1	.....	.....	16.2	.....	21.4	28.7
No. of leaves	.....	3.0	.....	.....	5.3	.....	6.9	8.7
Dead or dying leaves	.....	1.1	.....	.....	2.6	.....	6.2	8.5

* Measured from ground line to tip of leaf uppermost when all were extended.

At 66 days the average numbers of elongated or elongating internodes per plant with decreasing watering frequency were 1.4, 1.3, 0.2, and 0, the completely mature units averaging 58, 45, and 43 mm. in length, respectively. Comparable data at 119 days are 5.1, 3.0, 1.25, and 0.5, with lengths of 72, 46, and 42 mm. No elongated internodes were completely mature in the 20's. The usual lowest internode to elongate (considering the mesocotyl as the first internode) was the seventh in the 3's, seventh or eighth in the 6's, and eighth or ninth in the 12's and 20's. Most 20's, so far as could be determined, thus had one or two more unelongated internodes per plant than had the 3's. This change in position for internodal elongation of the culm, apparently due (at least indirectly) to deficient water supply, is probably the most fundamental morphogenetic effect induced by the treat-

ment, for it seems improbable that the observed differences are the result of inadequate sampling. It should be noted that maturation of the leaf base and basal internode regions was complete in nearly all plants examined only two or three nodes below the apex of the axis in all series and ages.

The successive dying of the four or five lowest foliage leaves, which occurred in all series in 4–8 weeks, was apparently a normal process, probably caused by the rupture of the vascular strands in their bases through the development of adventitious roots and axillary tillers, and did not seem due directly, except possibly in the 20's, to any deficit in external water supply. The death of leaves above this level (table 3), and of those of the secondary axes (table 5), was hastened in all series in the last 6 weeks by the increasing aridity of aerial environment and by the increasing difficulty for the larger plants to maintain a favorable water balance with the amount supplied. In the 3's and 6's especially, the developing tillers undoubtedly diverted water necessary to maintain the upper leaves on the elongating primary axis. This increasing deficit in upper portions of the plants in these series may likewise have been responsible for their failure to initiate spikes at an age and apparent stage of development when such initiation might have been expected. It is known (**2, 11**) that individuals of this perennial will flower within the first four months of growth; but the complex interactions of age of plant, photoperiod, and temperature known to affect reproductive activity in certain grasses (**10**) are almost completely unknown for this species. The terminal growing point was still alive in all plants at the close of the experiment.

Tiller development

Axillary bud primordia successively appeared in the axils of all primary leaves except the scutellum, coleoptile, and the upper two or three foliage leaves in the 119-day 3's and 6's. Tables 4 and 5 show the total number of buds, the size attained, the number of visible leaves, and the death of successive leaves in the various tillers formed from these buds, in relation to age and watering frequency. The data emphasize the relatively greater influence of water supply upon size than upon the numbers of organs differentiated, also noted for the primary axis. In all series the ratio of leaves per tiller to its length shows a tendency to decrease with increase in nodal position. This is most obvious on the older tillers where a larger proportion of both leaves and internodes had ceased elongating. Occasional plants appeared in all series in which the basal axillary bud did not elongate. These account in part for the lesser average height of the first tiller, as compared with the second or third, in the older plants. These basal buds often assumed a horizontal position, in contrast to the apogeotropic tendencies of all others; but at the close of the experiment it was impossible to determine whether they would remain dormant or become rhizomatous. Relatively short rhizomes

are characteristic of this perennial species. It was likewise undetermined whether or not this failure of basal buds to enlarge was related to depth of planting, as it seems to be in wheat (4).

The largest tillers in each series at 119 days occurred most commonly in the axil of the second, occasionally of the third, foliage leaf (fourth and fifth nodes). They averaged 46.3, 29.9, 20.3, and 12.1 cm. in length, with leaf numbers of 9, 8.5, 7.9, and 7.7 in order of decreasing water supply. At the same age, and in the same order, the average numbers of primary tillers over 15 mm. in length were 5.4, 4.7, 4.2, and 3.0. Thus in the 3's and 6's practically all buds borne on the basal portion of the axis had expanded, in contrast to less actual and much lower percentage development in the 12's and 20's with their greater number of basal nodes. In the layman's language the latter had failed to "stool out well," due primarily to a retardation in growth. Internodal elongation in primary tillers had occurred after 119 days in 3's, 6's, and 12's, an average of 3.0, 1.4, and 0.7 tillers per plant showing elongation in the respective series. Position of the first elongating internode in the secondary axis decreased from 6–9 to 4–6 in passing from the first to fourth primary tiller. Positive correlation of its position with water supply is not possible with the data now available.

TABLE 4

TOTAL NUMBER OF PRIMARY, SECONDARY, AND TERTIARY BUDS

Series	Date and age in days						
	Dec. 9 (18)	Dec. 17 (26)	Dec. 21 (30)	Jan. 2 (42)	Jan. 6 (46)	Jan. 26 (66)	Mar. 19 (119)
3's							
Primary	0	.....	0.7	3.3	.....	6.0	7.4
Secondary	0	.....	0	0.6	.....	15.2	25.0
Tertiary	0	.....	0	0	.....	2.2	24.8
6's							
Primary	0	.....	0.4	2.8	.....	5.4	7.4
Secondary	0	.....	0	0.2	.....	9.6	21.8
Tertiary	0	.....	0	0	.....	0.9	13.7
12's							
Primary	0	.....	0.1	2.9	.....	5.0	7.1
Secondary	0	.....	0	0	.....	4.5	17.6
Tertiary	0	.....	0	0	.....	0	9.0
20's							
Primary	.....	0	.....	.....	2.4	4.1	6.6
Secondary	.....	0	.....	.....	0	2.2	12.8
Tertiary	.....	0	.....	.....	0	0	0

The retardation in differentiation due to water deficit shows most clearly in the data on secondary and tertiary buds (table 4). Secondary buds were regularly

TABLE 5

LENGTH OF TILLERS, AND TOTAL NUMBERS OF LEAVES AND OF DEAD AND DYING LEAVES, ON SUCCESSIVE PRIMARY TILLERS

SERIES	DEC. 21	JAN. 2	JAN. 6	JAN. 26	MAR. 19
	1ST TILLER				
3's					
Length, mm.	5*	43	.....	282	337
No. of leaves	1.4*	4.9	.....	8.8	10.2
Dead or dying leaves	0	0	.....	0.0	9.2
6's					
Length, mm.	3	27	.....	116	134
No. of leaves	1.0	3.6	.....	7.1	8.8
Dead or dying leaves	0	0	.....	3.8	8.3
12's					
Length, mm.	3	14	.....	44	86
No. of leaves	1.0	3.5	.....	6.1	6.9
Dead or dying leaves	0	0	.....	2.1	5.8
20's					
Length, mm.	.....	.....	7.7	50	60
No. of leaves	.....	.....	3.6	6.0	7.3
Dead or dying leaves	.....	.....	0	1.1	6.9
	2ND TILLER				
3's					
Length, mm.	5	31	.....	311	446
No. of leaves	1.5	3.9	.....	7.4	9.6
Dead or dying leaves	0	0	.....	5.0	8.6
6's					
Length, mm.	0	29	.....	250	283
No. of leaves	0	3.0	.....	6.3	8.6
Dead or dying leaves	0	0	.....	4.2	8.3
12's					
Length, mm.	0	18	.....	103	175
No. of leaves	0	2.8	.....	5.9	8.4
Dead or dying leaves	0	0	.....	2.0	8.0
20's					
Length, mm.	.....	.....	16	68	111
No. of leaves	.....	.....	3.0	5.2	7.9
Dead or dying leaves	.....	.....	0	1.1	7.1

* Based on average total tillers present in ten plants.

TABLE 5—*Continued*

Series	Dec. 21	Jan. 2	Jan. 6	Jan. 26	Mar. 19
	3rd tiller				
3's					
Length, mm.		7		251	408
No. of leaves		1.8		6.4	8.0
Dead or dying		0		4.4	7.2
6's					
Length, mm.		8		211	236
No. of leaves		1.4		5.3	8.0
Dead or dying leaves		0		2.7	7.1
12's					
Length, mm.		5		68	194
No. of leaves		1.3		4.6	7.6
Dead or dying leaves		0		0.8	7.0
20's					
Length, mm.			4	32	101
No. of leaves			1.1	3.9	6.9
Dead or dying leaves			0	0.1	6.4
	4th tiller				
3's					
Length, mm.		3		191	178
No. of leaves		1.0		5.0	7.0
Dead or dying leaves		0		1.8	6.8
6's					
Length, mm.		3		115	184
No. of leaves		1.0		4.4	6.2
Dead or dying leaves		0		0.5	5.6
12's					
Length, mm.				22	140
No. of leaves				3.3	6.2
Dead or dying leaves				0.1	5.6
20's					
Length, mm.				5	25
No. of leaves				1.3	3.8
Dead or dying leaves				0	2.7

TABLE 6

AVERAGE NUMBER PER PLANT, BY SIZE CLASSES AND CONDITION, OF ADVENTITIOUS ROOTS AND PRIMORDIA IN RELATION TO AGE AND WATER SUPPLY

Series	Length (mm.)	Date and age in days						
		Dec. 9 (18)	Dec. 17 (26)	Dec. 21 (30)	Jan. 2 (42)	Jan. 6 (46)	Jan. 26 (66)	Mar. 19 (119)
3's								
Living	50+	0.2	.....	0.8	2.2	.....	8.8	10.8
Living	20–50	0.4	.....	0.5	0.8	.....	4.4	0
Living	5–20	0.5	.....	0.6	1.6	.....	5.8	0
Living	5–	0.3	.....	0.7	1.6	.....	4.2	15.6
3's								
Dead	50+	0	.....	0	0.1	.....	0	0.8
Dead	20–50	0	.....	0.1	0.3	.....	0.6	2.4
Dead	5–20	0	.....	0.1	0.4	.....	0.4	6.0
Dead	5–	0	.....	0.2	1.4	.....	0.8	7.8
Total 3's living and dead		1.4	.....	3.0	8.4	.....	25.0	43.4
6's								
Living	50+	0	.....	0.5	2.2	.....	6.0	5.9
Living	20–50	0	.....	0.1	0.2	.....	0.1	0
Living	5–20	0	.....	0.4	0.3	.....	0.6	0.3
Living	5–	0.3	.....	1.0	0.4	.....	0	11.7
6's								
Dead	50+	0	.....	0	0	.....	0.4	0.3
Dead	20–50	0	.....	0.2	0.1	.....	0.6	1.1
Dead	5–20	0	.....	0.1	0.4	.....	1.0	7.6
Dead	5–	0	.....	0.1	1.3	.....	4.6	12.9
Total 6's living and dead		0.3	.....	2.4	4.9	.....	13.3	39.8
12's								
Living	50+	0	.....	0	2.1	.....	2.6	3.9
Living	20–50	0	.....	0	0.4	.....	0.4	0
Living	5–20	0	.....	0.5	0.2	.....	0.2	0
Living	5–	0	.....	0.5	0.9	.....	0.1	14.2
12's								
Dead	50+	0	.....	0	0	.....	0	0
Dead	20–50	0	.....	0	0	.....	0.3	0.4
Dead	5–20	0	.....	0	0.2	.....	1.5	3.1
Dead	5–	0	.....	0.4	0.1	.....	7.1	5.3
Total 12's living and dead		0	.....	1.4	3.9	.....	12.2	26.9
20's								
Living	50+	.....	0	.....	.....	0.1	1.5	1.7
Living	20–50	.....	0	.....	.....	0	0	0
Living	5–20	.....	0	.....	.....	0	0	0
Living	5–	.....	0.5	.....	.....	0	1.3	9.5
20's								
Dead	50+	.....	0	.....	.....	0	0	0
Dead	20–50	.....	0	.....	.....	0	0.4	0
Dead	5–20	.....	0	.....	.....	0.7	1.0	1.6
Dead	5–	.....	0	.....	.....	2.9	5.5	7.3
Total 20's living and dead		.....	0.5	.....	.....	3.7	9.7	20.1

differentiated in the axils of all secondary leaves except the prophylls, the lowest three or four (occasionally all) leaves of the lowest tillers which failed to elongate, and some of the uppermost leaves on elongated secondary axes. Buds were sometimes found in the axils of prophylls, especially those on the higher secondary axes. No positive correlation of this occurrence with water supply was evident. At the end of 4 months the number of secondary tillers over 15 mm. in length was low, averaging only 5, 2.6, and 2.9 in the 3's, 6's, and 12's, respectively. Tertiary buds grew only slightly in any of the plants possessing them, and buds of the fourth order were found rarely in the 3's and 6's at 119 days. The greatest number of tertiary buds occurred on the second primary tiller, in accord with its usual greater size and with the usual location of the largest secondary tillers on each plant.

Secondary root system

The apparent normal plan of adventitious root initiation in this species involves the differentiation of one primordium at the coleoptilar or second node, at the level of insertion of the coleoptile, and on the same side of the axis and in the same plane as the line along which the coleoptile splits. Successive nodes, up to the first or second below the first elongating internode, normally produce a pair of primordia on the side of the axis opposite the bud insertion at the same node. These usually emerge just at or slightly below the level of leaf insertion, and above and slightly to either side of the next lower bud, so that in development they grow down on opposite sides of, and sometimes almost surround, this secondary axis. Roots from the third node usually surround the split coleoptile in similar fashion. An additional pair of primordia are normally initiated slightly above the base of the first elongating internode or on the internode just below it when none are produced on the last node of the abbreviated stem. These usually emerge on the same side of the axis as the axillary bud, but slightly above and to either side of the latter. A third primordium is sometimes found at the same level but directly opposite the point of insertion of the bud. To simplify tabulation, these last two or three roots are listed in table 7 under the number of the node next above them.

The actual number of root primordia produced by the primary axis rarely agrees with this normal plan. Two tendencies, sometimes working together and sometimes in opposite directions, seem to be superimposed upon this normal development. With greater or less supplies of water, a greater or less number of primordia tend to be associated with any node, as may be seen by comparing the totals in the different series for the third, fourth, and fifth nodes of the primary axis (table 7). Numbers initiated on the higher nodes, however, and on secondary axes as well, in individual plants vary inversely with the number of primordia which have already matured into functional roots. The effect of this latter tend-

TABLE 7

ADVENTITIOUS ROOT AND PRIMORDIA NUMBERS, CONDITION, AND DISTRIBUTION ON AXIS, BY SIZE CLASSES, ON 119-DAY PLANTS

SERIES	LENGTH (MM.)	PRIMARY STEM AXIS: ROOTS PER NODE									SECONDARY STEM AXES: ROOTS PER AXIS						TERTIARY AXES: TOTAL ROOTS	GRAND TOTAL
		NODE 2*	3	4	5	6	7	8	9	TOTAL	AXIS 1	2	3	4	5	TOTAL		
3's																		
Living	50+	0.8	1.4	0.8	1.4	1.2	0.2	0	0	5.8	1.8	2.0	0.8	0	0	4.6	0.4	10.8
Living	20–50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living	5–20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living	5–	0	0	0	0	0	0.2	0.6	0	0.8	3.2	2.2	2.6	2.2	0.8	11.0	3.8	15.6
3's																		
Dead	50+	0	0	0	0.2	0.2	0	0	0	0.4	0.2	0.2	0	0	0	0.4	0	0.8
Dead	20–50	0	0.2	0.8	0.6	0	0.2	0	0	1.8	0	0.4	0	0	0	0.4	0.2	2.4
Dead	5–20	0	0.2	0.6	0.6	0	0.4	0	0	1.8	1.8	0.8	0.8	0.4	0	3.8	0.4	6.0
Dead	5–	0.2	0.8	0.4	0	0.4	0.8	0.6	0	3.2	1.6	1.2	0.6	0.6	0.2	4.2	0.4	7.8
Total living and dead		1.0	2.6	2.6	2.8	1.8	1.8	1.2	0	13.8	8.6	6.8	4.8	3.2	1.0	24.4	5.2	43.4
6's																		
Living	50+	0.3	0.5	0.9	0.8	0.5	0.3	0	0	3.3	0.8	1.1	0.6	0	0	2.5	0.1	5.9
Living	20–50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living	5–20	0	0	0	0	0	0	0	0	0	0	0.1	0.1	0.1	0	0.3	0	0.3
Living	5–	0	0	0	0	0	0.4	0.8	0.7	1.9	1.1	2.0	2.3	1.5	0.3	7.2	2.6	11.7
6's																		
Dead	50+	0	0	0.1	0.2	0	0	0	0	0.3	0	0	0	0	0	0	0	0.3
Dead	20–50	0	0.1	0.3	0.3	0	0.2	0	0	0.9	0	0.2	0	0	0	0.2	0	1.1
Dead	5–20	0.4	0.5	0.7	0.7	1.6	0.3	0	0	4.2	1.0	1.6	0.4	0.2	0	3.2	0.2	7.6
Dead	5–	0.3	0.6	0.1	0.4	1.0	1.8	0.3	0.4	4.9	2.6	2.0	2.3	0.6	0	7.5	0.5	12.9
Total living and dead		1.0	1.7	2.1	2.4	3.1	3.0	1.1	1.1	15.5	5.5	7.0	5.7	2.4	0.3	20.9	3.4	39.8

* Coleoptilar node.

* Coleoptilar node.

TABLE 7—*Continued*

Series	Length (mm.)	Primary stem axis: roots per node									Secondary stem axes: roots per axis						Tertiary axes: total roots	Grand total
		Node 2*	3	4	5	6	7	8	9	Total	Axis 1	2	3	4	5	Total		
12's†																		
Living	50+	0	0.5	1.25	0.875	0.75	0	0	0	3.37	0.25	0.25	0	0	0	0.5	0	3.9
Living	20–50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living	5–20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Living	5–	0	0	0	0	0	0.63	1.75	0.125	2.5	2.13	3.13	3.0	1.88	0.125	10.25	1.5	14.2
12's†																		
Dead	50+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dead	20–50	0.125	0	0	0	0.25	0	0	0	0.38	0	0	0	0	0	0	0	0.4
Dead	5–20	0.375	0.25	0.25	0.625	0.5	0.25	0	0	2.25	0.25	0.5	0.125	0	0	0.875	0	3.1
Dead	5–	0.5	0.5	0.25	0.5	0.5	1.13	0.375	0	3.75	0.5	0.5	0.375	0	0	1.375	0.125	5.3
Total living and dead		1.0	1.25	1.75	2.0	2.0	2.0	2.13	0.12	12.25	3.125	4.375	3.5	1.875	0.125	13.0	1.625	26.9
20's																		
Living	50+	0	0.2	1.0	0.5	0	0	0	0	1.7	0	0	0			0	0	1.7
Living	20–50	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0
Living	5–20	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0
Living	5–	0	0	0	0	0.4	1.8	1.5	0.3	4.0	1.6	2.1	1.8			5.5	0	9.5
20's																		
Dead	50+	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0
Dead	20–50	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0
Dead	5–20	0.3	0.2	0.1	0.6	0.1	0.1	0	0	1.4	0.2	0	0			0.2	0	1.6
Dead	5–	0.7	1.3	0.7	1.0	1.5	0.5	0.2	0	5.9	0.6	0.7	0.1			1.4	0	7.3
Total living and dead		1.0	1.7	1.8	2.1	2.0	2.4	1.7	0.3	13.0	2.4	2.8	1.9			7.1	0	20.1

† Averaged from eight plants.

ency can be seen most strikingly in the totals for the sixth and seventh nodes in the 6's, and for the sixth node in the 20's (table 7). The higher average values for these nodes in these series as compared with the 3's and 12's were due almost entirely to the numbers of roots initiated at these levels in the first pair of series by one or two plants per pot which had thus far failed to establish any adventitious roots. The tendency was suppressed in the 20's to a greater extent than in the 6's, probably by the greater inadequacy of the water supply.

Because of the abbreviated character of the basal internodes of the primary stem, it was impossible, by dissection alone, to determine the nodal or internodal character of all the roots produced in excess of the normal. Those which seemed definitely to be internodal[2] were included with those emerging from the next higher node, in tabulation. No attempt was made to work out the plan, if any, in the position of these roots, nor in that of those produced on secondary and tertiary axes. The curved character and the exceedingly abbreviated basal internodes of the branch axes make such analysis extremely difficult. The first root to appear, and the one which most commonly became established, on the secondary shoots, emerged on the lower side of the stem, and thus at right angles to the plane of phyllotaxy, at the third or fourth node.

The rate of production of primordia and the total number produced naturally varied with the rate and degree of development of the shoots, as related to water supply, as may be seen from the totals in table 6, and in detail in table 7. Scatter charts, based on the analysis of individual plants, showed a strong correlation between totals of root primordia produced and development of tops, as expressed in total numbers of axillary buds. The various treatments had little apparent effect on this relationship. Correlation was less apparent between total bud numbers and total live roots over 50 mm. long, but was more evident, although still weak, when tillers over 15 mm. long were correlated with the same root condition. This possibly indicates again that water supply for the entire plant, if it is related to the number of functional roots, has less effect upon numbers of parts differentiated in the top than upon their size. In this connection, no apparent correlation was found in any series between the size and development of tillers at any specific node and the number of functional roots established at the same or adjacent nodes. This might have been expected in view of the known irregularity (1) in the connection of the vascular elements of roots and buds in certain grasses with those of their parent axis at the same nodal level.

The ultimate development of any primordium seems to be intimately connected with the water supply, both in the plant and in the environment, during a varying length of time following its initiation, and with its position above the soil surface. The data in table 7 show the condition, when the plants were 4

[2] ARBER (1) notes rare exceptions to the common interpretation that all adventitious grass roots are nodal in origin.

months old, of the roots which had been initiated successively at the different nodes of the primary axis. As an example, initiation of the root at the coleoptilar node probably took place in all series when the plants were about 15 days old. In the 3's a continuously moist soil surface and an abundance of water in the plant allowed growth to proceed promptly in most cases, and new primordia began to form at the third node, resulting at 18 days (on December 9) in the live root condition shown in table 6. Table 7 shows that eight of the ten plants examined at 119 days in this series had functional roots on the coleoptilar node. In the 6's and 12's at 18 days, with no water added for 6 and 12 days, respectively, the primordia were so small as not to be counted in most cases, as shown by the data in table 6 for December 9. These primordia responded immediately to the addition of water on this date, some of them reaching lengths of 10 mm. over night. But the rate of soil drying, downward, exceeded their rates of growth, and only three of the 6's and none of the 12's show roots established from this node (table 7). In the 20's the equivalent primordia were delayed in their growth even longer following initiation, although some had protruded through the stem tissue about 1 mm. at 26 days. They all grew immediately upon watering at this time, some reaching lengths of 5 mm. within 6 hours; but in spite of this rapid growth, none succeeded in becoming established before the surface soil again became dry.

In 6's, 12's, and 20's, each application of water invariably resulted in immediate elongation of the small primordia already initiated, until the plants were about 8–10 weeks old. After this, and earlier in the concurrent studies on drought effect and recovery, a decided lag (often 2 or 3 days) occurred after watering before there was much evidence of growth in primordia already initiated. The latter condition was also found in older plants of all series where root tips, deep in the soil, had ceased to grow in the later stages of a watering interval and had become differentiated almost to the tip. This lag in resumption of growth by the primordia of older plants, which likewise seem to differentiate to the tip if growth is delayed, increases their difficulty in establishment in a soil which rapidly dries after water is added, and accounts in part for the large number of dead roots under 20 mm. long in all series on March 19.

The 3's show a lower number of established roots than might be expected. Death losses to account for this were due probably to the possible lack of aeration during late December and January, as mentioned previously, possibly accompanied by fungus or bacterial invasion. The death of roots initiated at a later period in the 3's, and that of all roots in the other series, seemed to be due to desiccation. The somewhat higher percentage of living long roots in the 20's than was anticipated was due entirely to the coincidence of a 4-day period of cloudiness following the watering at 46 days, allowing some of the primordia from the third, fourth, and fifth nodes to become established before the soil surface became dry (table 7).

Discussion

The more obvious effects of varying water supply upon the size and development of *Bouteloua curtipendula* need little interpretation. The primary root showed, under the relatively mesic conditions of the early part of this experiment, the rapid and deep penetration characteristic of many species adjusted to semiarid or arid environments (**2**), so necessary for their successful establishment. Its future development and persistence seem closely related to the establishment of adventitious roots. It apparently becomes nonfunctional in plants where abundant and frequent renewal of water supply allows the latter to develop, but persists for at least 4 months as the sole or most important part of the absorbing system in the drier series where few or no nodal roots become functional. No assumptions are made as to the causes of death or persistence, except in the 3's, where death may have been due to inadequate aeration. It has been reported that winter wheat (**8**) may complete its life cycle with only the seminal roots established; and McCALL (**9**) states that, unless destroyed in some way, the primary root system remains functional throughout the life of the wheat plant. The latter would not be true in perennial side-oats grama, and it is doubtful whether, under natural conditions, its mesocotyl is strong enough to withstand, alone, the mechanical stresses imposed upon plants of the size attained by the 20's at the end of 4 months (fig. 1), even though sufficient water and nutrients might be absorbed and transmitted by the primary root system to maintain life.

The position of the coleoptilar, or first crown, node at the ground surface (probably a usual condition in nature with shallow planting) would seem to be disadvantageous for a species growing in arid environments, for each successively formed nodal root primordium to become functional must surmount the difficulties encountered in growing through an inch or two of surface soil which is dry much of the time. Notwithstanding the relatively rapid growth rates of such primordia when water became available, it apparently required about 3 consecutive days of soil surface wetness for successful establishment of such roots. Such conditions are occasionally part of the natural environment of this species during the critical periods following its germination, but a very high percentage of seedlings in nature probably do not survive because of failure in nodal root establishment, especially in widely spaced bunchgrass types. In older plants the increasingly mesic conditions provided by the plant's own shade should aid establishment of roots from tillers, while in propagation from rhizomes this situation probably is met easily. Development has not been traced this far, and no references have been found to the relative position of root origin on plants so produced.

The relative importance of primary and secondary root systems in the early life of the whole plant is obvious where one dies or the other fails in establishment.

No effort was made, however, to isolate the activities of each system, aside from death or persistence, under different conditions of water supply. Plants with no nodal roots were retarded in growth as compared with plants bearing functional adventitious roots in the same pots, but no obvious effect upon the relative size or differentiation of the primary axis as compared with the tillers was noticeable in relation to the presence or absence of one or the other systems. Such effects have been recorded by KRASSOVSKY (5) in spring wheat, barley, and rye. She concluded that the primary stem is supplied mainly by the seminal roots; the tillers, by the nodal roots. Since retardation in floral initiation was found in both primary stems and tillers in the present experiment, as compared with a 124-day plant illustrated by WEAVER (11), the failure of the main axis to flower at this age probably is not related causally, or only indirectly, to the death of the primary roots in the wetter series.

No explanation is advanced for the greater number of unelongated basal internodes in the primary stems of the dry series as compared with the wet, nor for the tendency of root primordia to develop in greater numbers on the lower nodes of the axis in the wet series than in the dry. The tendency toward increased root primordia numbers on the higher nodes in any series where roots from the lower nodes have failed in establishment possibly is related to the mechanism responsible for the rapid development of adventitious roots by the shoots of certain germinating grass embryos deprived of their seminal roots (6). LA RUE did not state specifically, however, whether or not the number of roots produced by any specific portion of the axis in his experiments was greater than it would have been under normal development at a later date. Regardless of the causal mechanism, the increased production of primordia noted in the present experiment, if followed by successful establishment at a favorable time, should be of adaptive significance in compensating for the earlier failure.

Detailed discussion of the implications of the data from this experiment in relation to the topics suggested in the introduction will be deferred until experiments with this species in other partially controlled environments are completed.

Summary

1. The growth of seedlings of *Bouteloua curtipendula* in a coarse sandy loam soil, in 2-gallon crocks 9 inches deep, watered at intervals of 3, 6, 12, and 20 days, was followed for 4 months. All plants received supplementary illumination to provide a 16-hour photoperiod.

2. At the end of the experiment, plant height, and total numbers of: leaves and nodes, and elongated internodes on the primary axis; primary, secondary, and tertiary tillers; adventitious root primordia differentiated; primordia differentiated on lower nodes of primary axis; and living functional adventitious roots

all were correlated directly with the amount of water supplied, although to different degrees. Numbers of: mature basal unelongated internodes; basal nodes bearing roots; and root primordia on upper crown nodes were correlated inversely with water supply, to a limited degree.

3. Height or length, as measured to tips of leaves, of both primary and secondary axes, was much more affected by water supply than was the number of leaves, buds, or roots differentiated on corresponding axes.

4. No plants flowered, although primary culm elongation began in the 3's and 6's within 60 days and had begun in the 20's at 4 months.

5. Both size and numbers of axes of the third and fourth order were much lower in the drier series than in the wetter ones.

6. The primary root system developed rapidly, but consisted only of a single taproot and its branches. It became nonfunctional in most 3's and 6's within 2 months, but remained alive in 12's and 20's until the close of the experiment, and in some cases persisted as the only functional part of the root system.

7. Adventitious roots require approximately 3 consecutive days of surface soil wetness to penetrate sufficiently to become established. A tendency was noted for a relative increase in the numbers of primordia differentiated, at the upper crown nodes and on tillers, by plants on which no nodal roots had become established.

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FURTHER OBSERVATIONS ON FACTOR Z[1]

WILLIAM J. ROBBINS

(WITH FOUR FIGURES)

Introduction

In earlier publications (**2, 3, 4, 5**) it was proposed that an unidentified factor, called for convenience factor Z, favorably influences the spore germination, early mycelial growth, and gametic reproduction of *Phycomyces blakesleeanus*. Factor Z appeared to be present in extracts of many natural products, including those of potato tubers. ROBBINS and HAMNER (**5**) separated an extract of potato tubers into two fractions by treatment with charcoal. One fraction, Ca, was adsorbed on charcoal and eluted with ammoniacal acetone; the other, Dr, was the charcoal filtrate. Each fraction acted favorably upon the development of *Phycomyces* in a medium containing thiamin, but the effect of the two fractions when combined in suitable proportions was greater than would be anticipated from their effects when used singly. ROBBINS and HAMNER suggested that the potato extract contained at least two unidentified factors: Z_1 adsorbed by charcoal and eluted by ammoniacal acetone and Z_2 present in the filtrate of potato extract which had been treated with charcoal. In the present paper further observations on these factors are reported.

Material and methods

Two basic media were used. Solution 1 contained per liter: 50 gm. dextrose, 1.5 gm. KH_2PO_4, 0.5 gm. $MgSO_4 \cdot 7\ H_2O$, 0.5 mg. thiamin, and asparagine as indicated. Solution 2 contained per liter: 100 gm. dextrose, 15 gm. KH_2PO_4, 5 gm. $MgSO_4 \cdot 7\ H_2O$, 0.5 mg. thiamin, and asparagine as indicated. To each of these solutions the following trace elements were added: 0.005 p.p.m. B, 0.02 p.p.m. Cu, 0.1 p.p.m. Fe, 0.01 p.p.m. Ga, 0.01 p.p.m. Mn, 0.01 p.p.m. Mo, and 0.09 p.p.m. Zn. All inorganic chemicals were of chemically pure grade: the dextrose was Corn Products Company chemically pure; the thiamin was Merck's synthetic; the asparagine was purified by precipitation from alcohol. For liquid cultures, 25 ml. of solution were used in 125-ml. Erlenmeyer flasks; for agar cultures, 25 ml. of media in petri dishes 90 mm. in diameter and 10 mm. deep were used. All solutions were sterilized at 110° C.; the temperature of incubation was $25° \pm 1°$ C.; dry weights were determined by drying at 100° C.

[1] Assistance in this work was furnished by the personnel of Works Projects Administration, Official Project 65-1-97-23, W.P. 5.

Effect of Ca and Dr fractions on Phycomyces and a method for approximating factors Z_1 and Z_2 in solutions of unknown composition

Drops of a spore suspension of *Phycomyces* were placed on 25 ml. of solution 1 solidified with 1 per cent agar. A similar procedure was followed with the same medium to which the Dr fraction, the Ca fraction, or combinations of the two were added (table 1). After 12 hours of incubation at 25° C. the percentage of germinated spores was determined. The Ca fraction contained 5.08 mg. solids per ml., and 1 ml. represented the extract from 50 gm. of potato. The Dr fraction contained 241.6 mg. per ml., and 1 ml. represented the extract from 50 gm. of potato. The preparation of these two fractions has been described earlier (5).

TABLE 1

Effect of Ca and Dr fractions on percentage of spore germination by Phycomyces at 25° C. on agar medium of minerals, sugar, asparagine, and thiamin

Ml. of fraction Ca per 25 ml. of medium	Ml. of fraction Dr per 25 ml. of medium	
	0.50	None
0.50	50.4	6.4
0.25	36.3	2.7
0.05	27.7	2.5
None	18.6	1.5

On the plates containing sugar, mineral salts, asparagine, and thiamin the percentage of germination was 1.5. The addition to this medium of 0.5 ml. of the Ca fraction increased the percentage to 6.4; the addition of 0.5 ml. of the Dr fraction increased it to 18.6. When the Dr and Ca fractions were both present, however, the percentage of germination was 50.4, which is greater than might be anticipated from the effects of either fraction alone. These results are illustrated in figure 1. As may be noted, the fractions of the potato extract affected not only the percentage of germination but the growth of the germ tubes.

The appearance of the plates after 69½ hours of incubation at 25° C. is shown in figure 2. The beneficial effect of the various extracts persisted and influenced the early mycelial growth.

The effect of the combined fractions on germination at 25° C. compared with their effect when used singly suggested a method of determining the presence of factor Z_1 (fraction Ca) and factor Z_2 (fraction Dr) in extracts of unknown composition. The method consisted of determining the effect of various amounts of the un-

known alone and in combination with the Ca and the Dr fractions prepared from potato on the germination of *Phycomyces* at 25° C. on a basic agar medium of mineral salts, sugar, asparagine, and thiamin. If the unknown and the Dr fraction combined caused a markedly greater percentage of germination than either

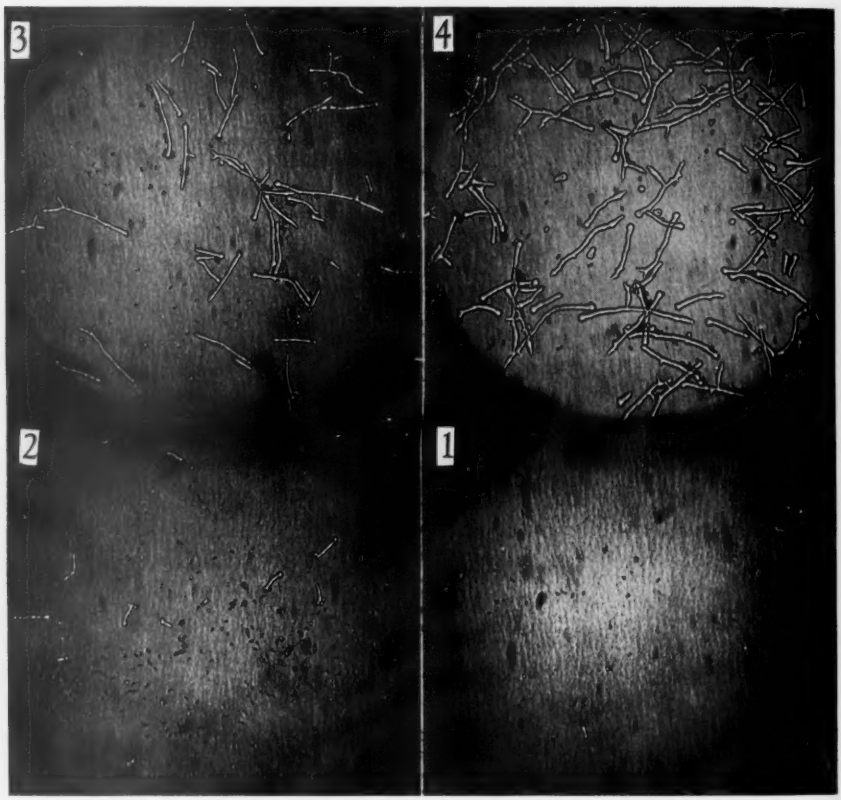

FIG. 1.—Germination of *Phycomyces* after 12 hours at 25° C. on: *1*, 1 per cent agar containing sugar, mineral salts, asparagine, and thiamin; *2*, same plus 0.5 ml. of Ca fraction per 25 ml. medium; *3*, same plus 0.5 ml. of Dr fraction per 25 ml. medium; *4*, same plus both fractions.

alone, it was considered presumptive evidence that the unknown contained effective amounts of the Z_1 factor and little or no Z_2. On the other hand, if the effect of the unknown and Ca fraction combined was much more than either alone, it would be evidence for the presence of factor Z_2 and little or no Z_1 in the unknown. If both factors were present in effective amounts, the unknown should favor germination considerably when used alone, and addition of either the Dr or the Ca fraction should not cause marked improvement.

The early mycelial growth of *Phycomyces* in liquid culture containing sugar, mineral salts, asparagine, and thiamin was benefited by the addition of suitable amounts of the Ca fraction or of the Dr fraction. Here again the combined fractions were much more effective than would be anticipated from their activity when used alone. This suggested another way of determining the presence of Z_1 or Z_2 in an unknown solution (table 2).

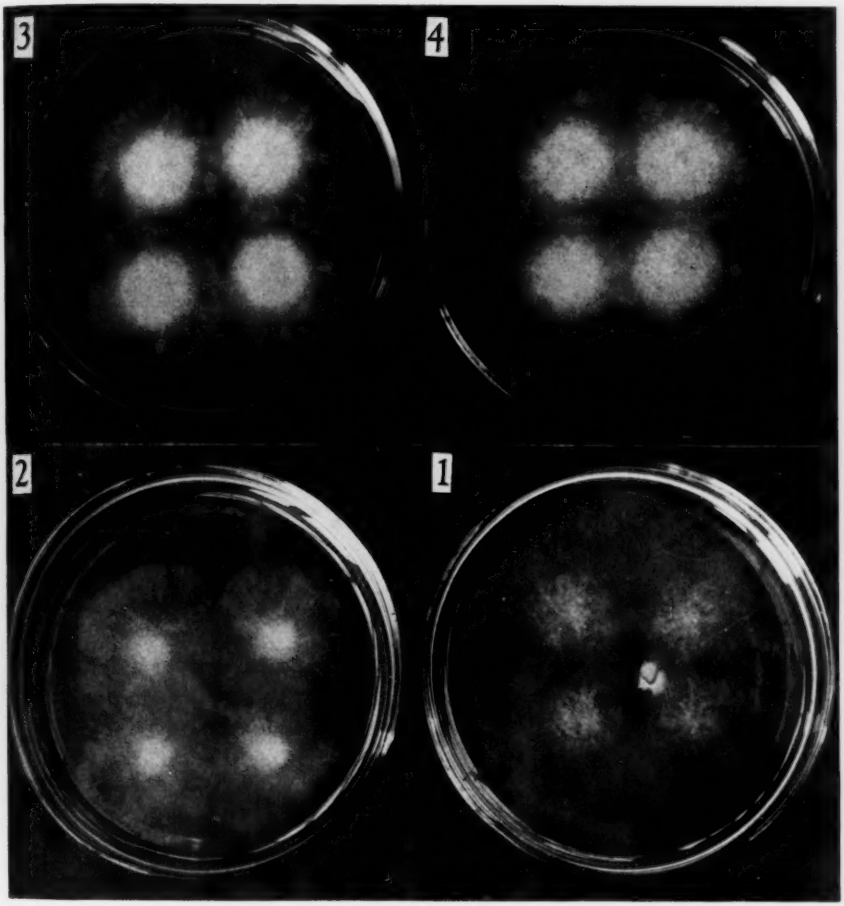

FIG. 2.—Growth of *Phycomyces* after 69½ hours at 25° C. on: *1*, 1 per cent agar containing sugar, mineral salts, asparagine, and thiamin; *2*, same plus 0.5 ml. of Ca fraction per 25 ml. medium; *3*, same plus 0.5 ml. of Dr fraction per 25 ml. medium; *4*, same plus both fractions. Each plate inoculated in four places with drop of spore suspension.

In this experiment a biotin concentrate from P. W. WEST containing 12.0 γ of biotin and 3.2 mg. of solids per milliliter was used in combination with the Dr fraction in the basic medium. The Ca fraction alone, the Dr fraction, and various combinations of the two were used at the same time. In the basic medium *Phycomyces* produced in a pair of flasks 0.2 mg. of dry matter at 25° C. in 71½ hours. The addition of 0.05 ml. of fraction Ca increased the yield to 1.3 mg. and 0.5 ml. to 3.7 mg. The addition of 0.5 ml. of Dr resulted in 31.5 mg. dry weight

TABLE 2

EFFECT OF CA FRACTION AND BIOTIN CONCENTRATE IN COMBINATION WITH DR FRACTION UPON DRY WEIGHT OF PHYCOMYCES PER CULTURE PRODUCED IN 71½ HOURS AT 25° C. LARGEST AMOUNT OF BIOTIN CONCENTRATE CONTAINED 0.2 γ BIOTIN. BASIC MEDIUM TO WHICH ADDITIONS WERE MADE CONTAINED MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

ML. OF BIOTIN CONCENTRATE OR CA FRACTION ADDED PER 25 ML. OF MEDIUM		ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM	
		0.50	NONE
0.166	biotin concentrate	27.7 mg.	.....
0.083	biotin concentrate	31.2	.....
0.0083	biotin concentrate	29.5	.....
0.00083	biotin concentrate	27.4	.....
0.000083	biotin concentrate	25.3	.....
None		31.5	0.2 mg.
0.50	fraction Ca	84.2	3.7
0.25	fraction Ca	68.8	2.1
0.05	fraction Ca	50.6	1.3

of mycelium (table 2). When the two fractions were combined the weights of the mycelium produced were 50 mg. or more. However, none of the combinations of the biotin concentrate with the Dr fraction gave yields higher than the Dr alone. It seems reasonable to conclude that the biotin concentrate in the amounts used contained little of the Z_1 factor, and that biotin and the Z_1 factor are not identical.

A similar procedure for determining the presence of Z_1 or Z_2 based on the effects on gametic reproduction at 25° C. of the Ca and Dr fractions alone and combined has also been used in some instances.

Experimentation

PRESENCE OF FACTOR Z IN PEPTONE.—Neopeptone was found to be superior to the potato extract in stimulating germination and early mycelial growth of *Phycomyces* in media which contained thiamin. This is illustrated in the following experiments.

An extract of potato, P_2, the preparation of which was described earlier (5), was

added in the amounts given in table 3 to solution 1 containing 1 per cent Difco agar and 2 gm. asparagine per liter. Neopeptone was also used in various quantities to supplement the basic medium. The germination of spores of *Phycomyces* on the basic medium and the media supplemented with potato extract or peptone was determined as already described. A comparison of the germination in the presence of the peptone with that on the media containing potato extract shows that the former was considerably more beneficial than the latter. For example, the addition of 0.0125 gm. of peptone per plate increased the percentage of germination to 73.8, while the addition of 0.015 gm. dry matter of the potato extract increased the germination to 4 per cent as compared with 0.4 per cent in the basic medium, which was supplemented with thiamin alone (table 3).

TABLE 3

EFFECT OF POTATO EXTRACT AND PEPTONE ON GERMINATION OF SPORES OF PHYCOMYCES AT 25° C. ON AGAR MEDIUM OF MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

Dry matter in potato extract added per 25 ml. of medium (gm.)	Percentage germination	Neopeptone added per 25 ml. of medium (gm.)	Percentage germination
0.155	90.0	0.25	85.3
0.078	72.5	0.10	93.3
0.031	38.5	0.05	91.9
0.015	4.0	0.025	82.0
0.0077	2.2	0.0125	73.8
0.0015	0.7	0.0025	8.1
0.0000	0.4	0.00025	1.1
		0.00000	0.4

The early growth of the mycelium also showed the greater benefit from peptone (table 4). In this instance the potato extract (P_2) or peptone was added to 25-ml. quantities of solution 1 containing 2 gm. asparagine per liter in 125-ml. Pyrex flasks. The experiment was performed in duplicate. After 72 hours of incubation at 25° C. the dry weights of the mycelium were determined. Even 1 mg. of peptone per 25 ml. of medium increased the growth to 15.3 mg. as compared with 1.7 mg. in the solution supplemented with thiamin only. A maximum of 119.1 mg. was secured with 0.01 gm. of peptone per 25 ml. of medium. The largest amounts of peptone and of potato extract which were used decreased growth.

Judging from the effectiveness of peptone in promoting germination and early mycelial growth, it contained both the Z_1 and Z_2 factors. Its action in combination with the Ca or Dr fractions on early mycelial growth and on gametic reproduction was determined also. From these results, which will not be detailed here, it appeared that peptone is rich in factor Z_1 and less rich in Z_2.

SUBSTITUTE FOR POTATO DEXTROSE AGAR.—Because of the favorable effect on the growth of *Phycomyces* of a medium containing thiamin and peptone, it has been used in preference to potato dextrose agar for maintaining stock cultures of filamentous fungi. Many species of *Phytophthora*, *Pythium*, *Fusarium*, and of the Mucoraceae grow well upon the thiamin-peptone medium.[2] It is simpler to prepare than potato-dextrose agar and has the further advantage of being transparent. No systematic attempt has been made to test groups of organisms upon it or to vary the constituents to determine their optimum proportions. It may not be so favorable for gametic reproduction as other media which can be devised, but its usefulness as a stock medium recommends it.

TABLE 4

EFFECT OF POTATO EXTRACT AND PEPTONE ON 72-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN LIQUID MEDIUM OF MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

DRY MATTER IN POTATO EXTRACT ADDED PER 25 ML. OF MEDIUM (GM.)	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)	NEOPEPTONE ADDED PER 25 ML. OF MEDIUM (GM.)	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)
0.31	87.4	0.2	15.0
0.124	129.1	0.1	27.3
0.062	113.8	0.05	95.1
0.031	88.9	0.02	119.1
0.0124	54.1	0.01	103.1
0.0062	27.8	0.005	57.8
0.0031	8.9	0.001	15.3
0.00062	2.3	0.0000	1.7
0.00000	1.7		

PRESENCE OF Z FACTORS IN AGAR.—The presence of the Z factor in agar has been previously reported (**2**). It was found possible to remove the Z factor in part by extracting the agar with methyl alcohol or aqueous pyridine. The effect of an agar extract upon the early growth of *Phycomyces* in a solution supplemented with thiamin is illustrated by the following experiments.

The agar extract was prepared[3] as follows: 424 gm. of Difco agar was treated in a percolator with 4 liters of 5 per cent pyridine, and the agar then washed with 2 liters of water and 4 liters of methyl alcohol. The brownish colored filtrates were combined, evaporated to small volume, and enough methyl alcohol added to make approximately 66 per cent. The precipitate was removed and the liquid again evaporated to small volume and treated with alcohol as before. The alcoholic liquor was evaporated to small volume and made up with distilled water to 200 ml.

[2] This medium contained per liter: 50 gm. dextrose, 2 gm. asparagine, 1.5 gm. KH_2PO_4, 0.5 gm. $MgSO_4 \cdot 7\ H_2O$, 0.1 mg. thiamin, 0.4 gm. peptone, and 1 per cent agar.

[3] The assistance of FREDERICK KAVANAGH in preparing this extract is acknowledged.

It contained 6.53 per cent solids, and 1 ml. represented the extract from 2.15 gm. of air-dry agar.

EFFECT OF AGAR EXTRACT ON GROWTH.—Various quantities of the agar extract were added to 25 ml. of solution 1 containing 2 gm. asparagine per liter or to the same solution without asparagine. The liquid was inoculated with a spore suspension of *Phycomyces* and incubated at 25° C. Dry weights were determined at the end of 72 hours (table 5). The experiment was performed in duplicate. Only a trace of mycelium had developed in 72 hours in the solution with no agar extract. The addition of 0.1 ml. of agar extract containing 6.53 mg. of solids resulted

TABLE 5

EFFECT OF AGAR EXTRACT ON 72-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN MEDIUM OF MINERAL SALTS, SUGAR, AND THIAMIN WITH 2 GM. ASPARAGINE PER LITER OR WITH NO ASPARAGINE

ML. OF AGAR EXTRACT ADDED TO 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)	
	MEDIUM WITH ASPARAGINE	MEDIUM WITH NO ASPARAGINE
None	Trace	.....
0.1	5.0	.....
0.2	15.0	.....
0.4	15.6	.....
0.8	15.5	.....
1.2	20.9	3.1
1.6	20.9	3.6
2.0	14.4	4.0

in the production of 5 mg. of dry mycelium. Larger amounts of agar extract were more beneficial, the maximum benefit being secured with 1.2 or 1.6 ml. of extract per 25 ml. of medium, 2 ml. resulting in less growth than was obtained with a smaller amount. The growth in a medium containing agar extract but no asparagine indicated that the amount of available nitrogen in the agar extract was small. Because of the beneficial effect of the extract on the early mycelial growth at 25 °C. in a basic medium of sugar, minerals, asparagine, and thiamin, it was concluded that the agar extract contained factor Z.

Although it appeared from this experiment that the agar extract contained factor Z, it seemed desirable to determine whether its beneficial action affected more than the early mycelial growth and what effect certain changes in the composition of the medium might have. Time curves were determined for the growth of *Phycomyces* in solution 1 with 5 gm. of asparagine per liter, in the same solution plus 1 ml. of agar extract per flask (25 ml. of medium), in the same solution plus 0.1 ml.

of agar extract, in solution 2 with 10 gm. of asparagine per liter, and in solution 2 plus 1 ml. of agar extract per flask. At the same time a few determinations were made of growth in solution 1, containing agar extract but no asparagine, no sugar, or no thiamin. The experiment was performed in duplicate and growth was measured by dry weights at 24-hour intervals (table 6; fig. 3). The beneficial effect of 1 ml. of agar extract was still evident after $8\frac{1}{2}$ days of growth. With the agar extract growth increased to a maximum of 416 mg. at $6\frac{1}{2}$ days and then decreased.

TABLE 6

EFFECT OF AGAR EXTRACT ON GROWTH OF PHYCOMYCES FOR VARIOUS PERIODS OF TIME IN SOLUTIONS OF MINERAL SALTS, SUGAR, ASPARAGINE, AND THIAMIN. SALT CONCENTRATION OF SOLUTION 2 WAS 10 TIMES THAT IN SOLUTION 1

PERIOD OF GROWTH IN DAYS	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)							
	SOLUTION 1 WITH 5 GM. ASPARAGINE PER LITER	PLUS 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	PLUS 0.1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO ASPARAGINE BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO SUGAR BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 1 WITH NO THIAMIN BUT 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM	SOLUTION 2 WITH 10 GM. ASPARAGINE PER LITER	PLUS 1 ML. AGAR EXTRACT PER 25 ML. MEDIUM
$1\frac{1}{2}$	0.0	1.6	0.0	.....	.....	.....	0.7	1.9
$2\frac{1}{2}$	0.5	8.5	3.9	.....	.....	.....	1.0	4.7
$3\frac{1}{2}$	14.3	63.1	28.0	.....	.....	.....	1.6	10.6
$4\frac{1}{2}$	141.3	228.1	206.3	.....	.....	.....	27.2	51.3
$5\frac{1}{2}$	260.3	391.2	345.1	.....	.....	.....	304.2	330.4
$6\frac{1}{2}$	292.6	416.1	363.6	.....	.....	.....	482.5	627.0
$7\frac{1}{2}$	300.1	391.2	365.0	7.4	1.2	14.7	415.1	711.6
$8\frac{1}{2}$	327.0	363.7	363.4	.....	.....	.....	621.8	734.4
$9\frac{1}{2}$	346.7	330.5	359.6	8.2	1.3	16.4	498.6	734.9
$10\frac{1}{2}$	320.6	318.2	359.5	.....	.....	.....	640.0	714.3
$11\frac{1}{2}$	349.0	317.9	364.4	.....	.....	.....	688.3	741.2
$12\frac{1}{2}$	.....	342.7	362.0	.....	.....	.....	676.2	693.3
$13\frac{1}{2}$	.....	322.8	363.3	11.0	1.0	20.2	649.8	655.0

Without the agar extract growth increased for $9\frac{1}{2}$ days until at that time growth in the medium with and without the agar extract was approximately the same. The 0.1-ml. agar extract was somewhat less effective than the 1-ml. extract.

Growth was less rapid for the first $4\frac{1}{2}$ days in solution 2 than in solution 1. Thereafter, however, the dry weight of the mycelium in solution 2 was considerably in excess of that in solution 1. The maximum dry weight (688.3 mg.) in solution 2 was attained in $11\frac{1}{2}$ days. I have no explanation for the irregularities in growth in solution 2 after $6\frac{1}{2}$ days (fig. 3). Growth in solution 2 containing agar extract was superior to growth in the same solution without extract for the entire period of the experiment. The agar extract contained approximately 0.2 millimicromole of thiamin per milliliter but very little available carbon or nitrogen.

In this experiment approximately half as much growth was obtained in solution 1 as in solution 2. It is not possible to say whether the increased buffer action re-

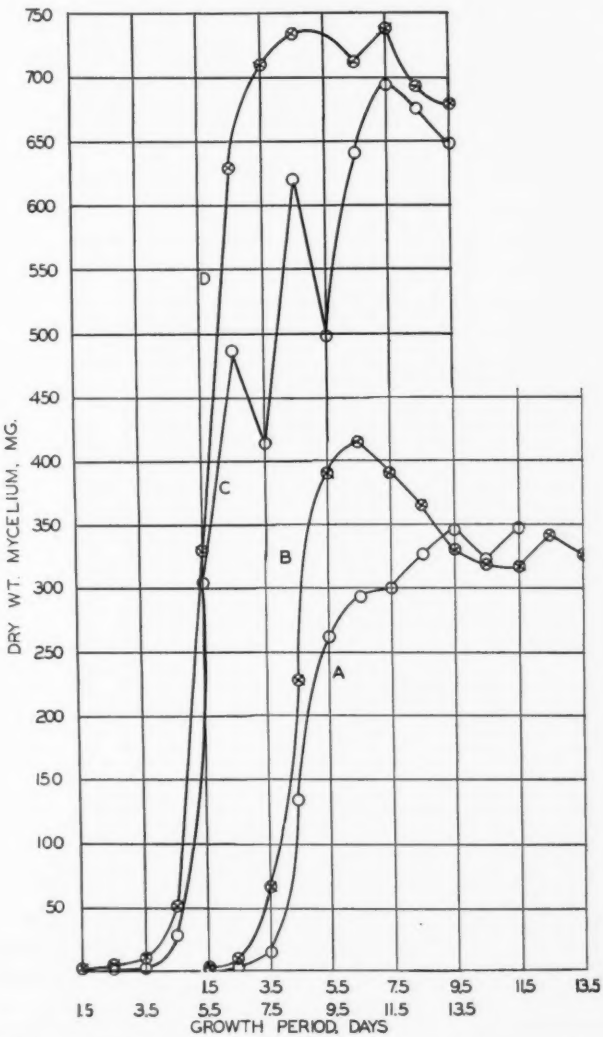

FIG. 3.—Time curves for growth of *Phycomyces: A*, in solution 1; *B*, in same solution plus 1 ml. agar extract per 25 ml. medium (both solutions contained 5 gm. asparagine per liter and excess thiamin); *C*, in solution 2; *D*, in solution 2 plus 1 ml. agar extract per 25 ml. medium (both solutions contained 10 gm. asparagine per liter and excess thiamin).

sulting from the greater quantity of phosphate, the greater quantity of minerals supplied, the greater amount of asparagine in solution 2, or some other factor, was responsible for this difference in total growth. It is probable that growth in solution 1 was limited by the amount of asparagine supplied. If this assumption is correct, then the beneficial action of the agar extract on growth in this solution was the result of some constituent of the agar other than thiamin, available nitrogen, or available carbon. The same statement would apply to the beneficial effect of agar extract on growth in solution 2.

In any event it seems probable that the beneficial effect of the agar extract is not because of its mineral content, since it was effective in both the weaker and stronger salt solution. It is possible that factor Z may beneficially influence the mature dry weight of *Phycomyces* at 25° C. as well as early mycelial growth, provided other factors (for example—thiamin, the nitrogen supply, sugar, or mineral salts) are not limiting. To determine this with certainty will probably not be possible until the constituents of factor Z are available in pure form.

In the experiment just described thiamin was supplied in luxus amounts. What effect would agar extract have on growth in solutions where thiamin is the limiting factor? This was determined by growing *Phycomyces* for 12 days in solution 2 with 10 gm. of asparagine per liter and various amounts of thiamin and in the same solutions plus 1 ml. of agar extract per 25 ml. of medium. The experiment was performed in duplicate. Figure 4 shows that the agar extract contained per milliliter between 0.1 and 0.2 millimicromoles of thiamin or its intermediates. The greater growth in the medium containing the agar extract and 0.1 or 0.2 millimicromoles of thiamin might be accounted for by the thiamin added. For amounts of added thiamin between 0.4 to 1.6 millimicromoles the differences in growth in the media with and without agar extract appear to be greater than can be accounted for by the thiamin content of the extract. Whether factor Z affects the dry weight of the mature mycelium of *Phycomyces* when thiamin is present in limited quantity is uncertain. Further investigation of this question should probably be postponed until the constituents of factor Z are available in pure form.

Each of the curves in figure 4 consists of two parts, one for the smaller amounts of thiamin and one for the larger amounts. Since little surface mycelium was formed in the solutions containing up to 0.4 millimicromoles of thiamin as compared with considerable surface and aerial mycelium with amounts of 0.6 millimicromoles thiamin and greater, the two parts of each curve may be related to this difference in growth habit.

FACTOR Z_1 IN AGAR EXTRACT.—The effect of the Ca and Dr fractions from potato on the action of agar extract was determined. It appeared that the agar extract contained considerable factor Z_1 and little Z_2. The addition of 0.5 ml. of the

Dr fraction to 25 ml. of medium produced 25.6 mg. of mycelium as compared with 2 mg. in the check solutions and 8.8 mg. in the solutions plus 1 ml. of agar extract (table 7). When 1 ml. of agar extract and 0.5 ml. of the Dr fraction were both present 76.2 mg. of mycelium was produced.

CATHODE AND ANODE LIQUORS FROM ELECTRODIALYZED AGAR.—Through the courtesy of C. E. MARSHALL, the cathode and anode liquors from a 30-gm. sample of shredded agar were obtained. Each of these liquors was neutralized and evaporated under reduced pressure to 100 ml. The concentrated anode liquor contained

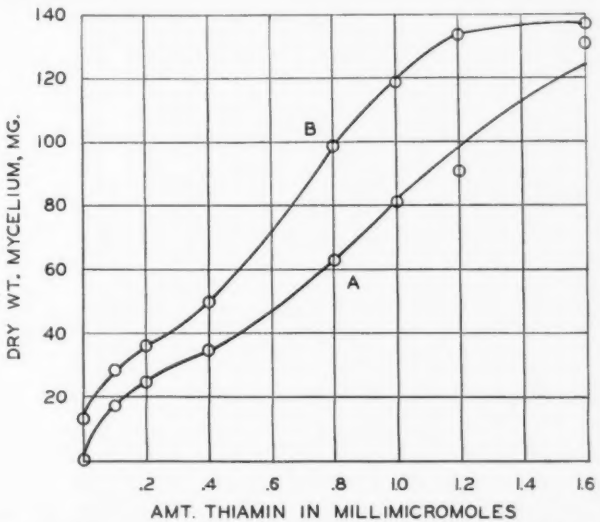

FIG. 4.—Growth of *Phycomyces* in solution 2 with 10 gm. asparagine per liter and various amounts of thiamin per flask: *A*, without agar extract; *B*, with 1 ml. agar extract per 25 ml. medium.

3.82 per cent dry matter; the cathode liquor, 1 per cent. Heated at 750° C., the ash in the anode liquor was 2.5 per cent, volatile dry matter 1.32 per cent; in the cathode liquor the ash was 0.27 per cent, volatile dry matter 0.73 per cent.

The effect of these liquors upon the early mycelial growth of *Phycomyces* was determined by addition of various quantities of each to solution 1 containing 2 gm. asparagine per liter. It was found (table 8) that the cathode liquor was beneficial and the anode liquor ineffective or somewhat toxic. The cathode liquor therefore contained appreciable amounts of factor Z, while the anode liquor contained little or none; in fact, a beneficial effect was obtained with 0.5 ml. of the cathode liquor which contained 5 mg. of dry matter. On a dry-matter basis the cathode liquor was about as effective as the agar extract previously used, but less effective than potato extract or neopeptone.

FACTOR Z_1 IN CATHODE LIQUOR.—When the cathode and anode liquors were used together as supplements to solution 1, the growth was intermediate between that obtained with the anode liquor or cathode liquor alone. When the anode liquor was combined with the Dr or Ca fraction from potato, no increase in growth over that with the liquor alone or the fractions alone was obtained. The cathode liquor in combination with the Ca fraction gave no increase, but with the Dr fraction the growth was much greater than with the cathode liquor or the Dr fraction alone. These results were taken to mean that the cathode liquor contained effective amounts of the Z_1 factor but little or no Z_2, and that the anode liquor lacked both.

TABLE 7

EFFECT OF CA AND DR FRACTIONS ON 70-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN SOLUTION 1 CONTAINING 2 GM. ASPARAGINE PER LITER AND AGAR EXTRACT

ML. AGAR EXTRACT ADDED PER 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)			
	NO ADDITION EXCEPT AGAR EXTRACT	0.5 ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM	0.5 ML. CA FRACTION ADDED PER 25 ML. OF MEDIUM	0.5 ML. CA FRACTION AND 0.5 ML. DR FRACTION ADDED PER 25 ML. OF MEDIUM
0.0	2.0	25.6	4.2	81.6
0.25	5.3	66.3	7.7	.....
1.0	8.8	76.2	9.7	.....

BIOTIN AND FACTOR Z_1.—ROBBINS and HAMNER (5) suggested that factor Z_1 might be biotin. The Ca fraction from potato, the anti gray-hair factor concentrate from MORGAN, and peptone—all of which contain factor Z_1 contain biotin also. By the use of *Ashbya gossypii* (6) I found per gram of dry matter in the Ca fraction of potato 1800×10^{-3} γ of biotin, in neopeptone 2100, in the anti gray factor concentrate 900, and in the Dr fraction from potato none. It appears, however, that the effective material in the Ca fraction of potato is not biotin.

Two biotin concentrates[4] were tested. The first was stated by WEST to contain 1.2 γ of biotin per milliliter and to have 0.3 mg. of dry matter per milliliter; the second was described earlier in this paper. Neither concentrate had much effect on spore germination and early mycelial growth of *Phycomyces* in solution 1. Neither concentrate affected the action of the Dr fraction from potato on the spore germination or early mycelial growth. From 0.00001 γ to 0.2 γ of biotin was used per flask containing 25 ml. of medium.

[4] I am indebted to Dr. P. W. WEST for these concentrates.

The coenzyme R concentrate was kindly furnished by F. E. ALLISON. Coenzyme R has been stated to be identical with biotin (**1, 7**). In contrast to the biotin concentrate from WEST, the concentrate of coenzyme R had a distinctly beneficial effect upon the early mycelial growth of *Phycomyces* in solution 1. The coenzyme R concentrate and the Dr fraction of potato combined were more beneficial than either alone. It was concluded that the coenzyme R concentrate contained some of the Z_1 factor.

TABLE 8

EFFECT OF CATHODE AND ANODE LIQUORS FROM AGAR ALONE AND IN PRESENCE OF EACH OTHER, THE CA OR DR FRACTIONS, ON 72-HOUR GROWTH OF PHYCOMYCES AT 25° C. IN SOLUTION OF MINERALS, SUGAR, ASPARAGINE, AND THIAMIN

ML. OF CATHODE OR ANODE LIQUOR ADDED PER 25 ML. OF MEDIUM	DRY WEIGHT OF MYCELIUM PER CULTURE (MG.)						
	CATHODE LIQUOR ADDED	ANODE LIQUOR ADDED	CATHODE AND ANODE LIQUOR ADDED	CATHODE LIQUOR AND 0.5 ML. DR FRACTION ADDED	CATHODE LIQUOR AND 0.5 ML. CA FRACTION ADDED	ANODE LIQUOR AND 0.5 ML. DR FRACTION ADDED	ANODE LIQUOR AND 0.5 ML. CA FRACTION ADDED
0	2.0	2.0	2.0	38.7	7.3	38.7	7.3
0.1	1.8	1.9	3.0	.....	.....	.....	.....
0.5	4.7	0.3	4.5	.....	.....	.....	.....
1.0	5.0	0.5	5.0	64.7	8.5	11.4	5.8
2.0	8.5	2.4	4.7	.....	.....	.....	.....

PANTOTHENIC ACID AND FACTOR Z_1.—The effect of pure synthetic calcium pantothenate[5] on the 72-hour growth of *Phycomyces* was determined in solution 1 with 2 gm. of asparagine per liter. Calcium pantothenate in amounts ranging from 0.01 to 10 γ per flask was used alone and in combination with the Ca and Dr fractions from potato. The calcium pantothenate was ineffective, and it was concluded that pantothenic acid is not identical with the Z_1 or Z_2 factors.

GLUTAMIN OR PARA-AMINO BENZOIC ACID AND FACTOR Z.—The effect of glutamin and of para-amino benzoic acid[6] on the 72-hour growth of *Phycomyces* was determined in solution 1 with 2 gm. asparagine per liter. Each compound was tested in amounts ranging from 0.001 to 5.0 mg. per flask and in combination with various amounts of the Ca and Dr fractions from potato. Neither compound appeared to be factor Z_1 or factor Z_2.

Discussion

The experimental results reported here support the conclusion of ROBBINS and of ROBBINS and HAMNER that unidentified growth factors influence the develop-

[5] Merck and Co. supplied this material.

[6] These preparations were kindly supplied by Dr. PAUL GYORGY.

ment of *Phycomyces* in a medium supplemented with thiamin. The results show further that a pyridine extract of agar and the cathode liquor from electrodialyzed agar contain appreciable quantities of factor Z_1 and little or no Z_2. Peptone was found to be rich in factor Z_1 and less rich in Z_2.

It is not possible as yet to identify either factor. Z_1 seems to belong in the vitamin B complex. It is probably not identical with biotin, pantothenic acid, vitamin B_2, pyridoxine (vitamin B_6), or thiamin. It is soluble in water and in aqueous alcohol or acetone; it is thermostable and migrates to the cathode in electrodialysis. It is strongly adsorbed by charcoal, from which it may be eluted by ammoniacal acetone. From evidence which will be presented elsewhere, one or both of these factors seem important for the development of other plants also. Since some of the growth substances of this general character (for example—pantothenic acid, thiamin, and pyridoxine) have been found necessary for both plants and animals, it is probable that factor Z_1 is a vitamin and may represent an addition to those already known.

As knowledge of the relation of vitamins and vitamin-like substances to plants increases, it seems evident that the relation is complex. Many such substances are required in the metabolism of a plant, just as they are in that of an animal, and the ability of a plant to synthesize one or more of them in sufficient amounts may determine the rapidity and completeness of its development. *Phycomyces* is unable to synthesize thiamin from the sugar, sulphate, and asparagine in the basic medium; unless supplied with that vitamin or its intermediates it is unable to grow. In the presence of thiamin, however, its development is influenced by its ability to make adequate quantities of two unidentified factors, Z_1 and Z_2. Important as thiamin is for the growth of *Phycomyces*, it is only one of many substances of similar function necessary for maximum and complete development. Most of these are synthesized in sufficient amounts by the organism. It is interesting to speculate on what the limiting factors for growth might be if all the vitamins and similar growth substances, as well as nutrients, foods, water, and oxygen, were supplied in luxus amounts.

Summary

1. Methods of estimating factors Z_1 and Z_2 in solutions of unknown composition are described.

2. Neopeptone was found to be rich in Z_1 and less rich in Z_2.

3. A pyridine extract of Difco agar was found to contain factor Z_1 but little Z_2. This extract influenced the mature dry weight of *Phycomyces* in the presence of excess thiamin.

4. The cathode liquor from shredded agar contained Z_1 but little or no Z_2. The anode liquor contained neither.

5. Z_1 was not found to be identical with biotin, pantothenic acid, glutamin, or para-amino benzoic acid. Z_2 was not found to be identical with glutamin or para-amino benzoic acid.

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INFLUENCE OF SULPHUR DEFICIENCY ON METABOLISM OF THE SUNFLOWER

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 520

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(WITH ONE FIGURE)

Introduction

There are few detailed studies of the effects of sulphur deficiency on the metabolism of plants. NIGHTINGALE (21) studied the effects in the tomato. The minus-sulphur plants were yellowish green in color and weighed much less than the plus-sulphur plants, although they were as tall as, or taller than, the latter. He explained this growth in height by the rapid proteolysis that was evidently going on in the sulphur-deficient plants. His analyses showed that these plants were high in soluble organic sulphur and nitrogenous compounds in comparison with the plants grown in a complete nutrient solution. NIGHTINGALE recognized that this proteolysis was abnormal, in that it took place in plants that were much higher in carbohydrates than were the plus-sulphur plants. Usually proteolysis is associated with a reduction in carbohydrates.

The situation in the soybean (5) was similar to that in the tomato, in that proteolysis and reutilization of the resulting compounds seemed to account for the elongation of the minus-sulphur plants. The proteolysis, however, was more in accord with that usually going on, for while the sulphur-deficient plants were high-carbohydrate, if all forms of carbohydrates were considered, they were much lower in the more available carbohydrates, reducing sugars and sucrose, than were the plus-sulphur plants.

The main object of the present work was to secure further data on the effects of sulphur deficiencies on metabolism, especially in regard to this theory of reutilization. Stem elongation of the minus-sulphur soybean plants was accounted for by proteolysis and the translocation of the resulting nitrogenous and sulphur compounds to the tips of the plants. But whole stems were analyzed in the case of the soybean. This was also true for the most part of NIGHTINGALE'S experiments with the tomato, although analyses were made for some of the sulphur fractions at different levels of the plant. In the present study the stem was analyzed at three levels, with the thought that if proteins of lower levels were being hydrolyzed and the resulting materials translocated to the tip, then positive gradients from below upward of the nitrogenous and sulphur fractions might be found.

No attempt is made to review the literature on sulphur. The literature reviews of the papers just referred to are adequate. McMURTREY (17) has reviewed the literature on sulphur, and WILLIS (35) has an extensive list of references with abstracts.

Material and methods

PRELIMINARY WORK.—Sulphur-deficient sunflower plants were grown for the first time in the spring of 1934, in connection with the work on the soybean (5). Decided symptoms of deficiency were obtained. The weights of tops and roots were taken and the top-root ratios calculated (5). No analyses were made.

During the spring of 1935, sunflower plants were grown according to the triangular method (11) to determine the best concentrations of the salts of the nutrient solution for growth of this plant. Some of them developed decided boron-deficiency symptoms, so in the autumn of 1935 and the spring of 1936 experiments were conducted to determine the best concentration of boron for the growth of the sunflower. The concentrations were extended into the toxic realm. The data have been reported (6).

TABLE 1

PARTIAL VOLUME MOLECULAR CONCENTRATION OF SALTS OF NUTRIENT SOLUTION

SULPHUR	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$	$MgCl_2$
Minus	0.0045	0.0090	.....	0.0045
Plus	0.0045	0.0090	0.0045	.....

FINAL PLAN.—Sunflower plants (*Helianthus annuus* L., variety Mammoth Russian) were grown from seed in pure quartz sand that had been washed thoroughly with distilled water. The plan of growing the plants was essentially the same as previously described (5), except that in the present work the natural day length was not supplemented by artificial light.

The preliminary work had shown that solution IR_2S_4 of the triangle (11) was best for growth of the sunflower. Table 1 gives its composition. Each solution has an osmotic pressure of one atmosphere. Boric acid at a concentration of 0.5 p.p.m. of boron was kept constantly in the nutrient solution, this being the concentration which the preliminary work showed was optimal. During the last 10 days of the experiment the concentration was increased to 0.75 p.p.m. Enough manganese chloride to give a concentration of 0.25 p.p.m. of manganese was also added.

The nutrient solution was applied in excess to the sand daily. The pots were flushed thoroughly twice a week with distilled water, and to the water was added iron as ferric chloride at a concentration of about 1 p.p.m. of iron.

The seed was planted April 4, 1939, and the plants harvested May 17. The plants were put in the darkroom the evening before and left until 8:00 A.M. the next morning, when the sampling was begun. At the time of harvest a few of the plants had begun to form heads; none of these were included in the samples. A number of the plus- and minus-sulphur plants were grown to maturity for data on the effects of sulphur deficiency on flower and seed production. These data are not reported in the present paper.

CHEMICAL METHODS

Fifty-three plus-sulphur and 135 minus-sulphur plants were taken for analysis. Only the stems were analyzed. In the case of soybean (5), sulphur deficiency affected the chemical composition of the stems much more than the other parts of the plant. In the present experiments each stem was divided into three sections of equal length, each section was cut into small pieces, and duplicate 100-gm. samples were taken of each.

Seventy per cent alcohol was used for the extraction instead of 80 per cent as in the previous work (5), to secure more complete extraction of the nitrogenous fractions. (STUART (28) found that, compared with water, 80 per cent alcohol gave very incomplete extraction of these compounds.) The tissue was covered with the alcohol, the alcohol heated to boiling on the steam bath, allowed to cool to room temperature, and filtered. Eight extractions were made, since tests with alpha naphthol showed that all the sugar was removed by this number of extractions.

CARBOHYDRATE FRACTIONS.—The carbohydrates were determined by essentially the same methods as previously described (5). In the permanganate titration the indicator ortho-phenanthroline was used (29). This decidedly increases the sharpness of the end point. Sucrose was determined after hydrolysis with invertase, as described by LOOMIS and SHULL (12). Their methods were also followed with slight modifications in the determination of starch and acid-hydrolyzable carbohydrates.

NITROGEN FRACTIONS.—The chief modification of the methods used previously (5) in the determination of the nitrogen fractions was that the ammonia was determined by the vacuum distillation method of VAN SLYKE (30). STUART (28) found that vacuum distillation was an efficient method of reducing the errors in the determination of amino nitrogen by the VAN SLYKE method (31). The presence of ammonia in the extract constitutes one of these errors. After the removal of ammonia by vacuum distillation, amino nitrogen was determined by the VAN SLYKE method; and as before, PHILLIPS' methods (23) were used in the determination of nitrates and amides.

Considerable difficulty was experienced in getting close duplicates in the nitrate determinations by PHILLIPS' original method, and the concentrations at the three

levels showed no regular trend. Possibly the difficulty might be accounted for by the splitting off of ammonia from organic nitrogenous compounds owing to the rather strong alkalinity of the extract. SESSIONS and SHIVE (**27**) got indications that even eighth-normal sodium hydroxide had a slight hydrolyzing effect on such compounds. Following PHILLIPS' (**24**) later suggestion, therefore, the nitrates were determined in a solution made tenth-normal with sodium hydroxide. After the acid digestion of the amides, the solution was nearly neutralized with 50 per cent sodium hydroxide, cooled, a slight excess of the sodium hydroxide added, and the amide nitrogen aerated off. No potassium carbonate was used. The solution left in the test tube was transferred to a beaker, neutralized with 1 : 1 sulphuric acid, and enough 50 per cent sodium hydroxide added to make 300 ml. tenth-normal. The solution was then transferred to a 800-ml. Kjeldahl flask, made up to a volume of 300 ml., and the determination completed in the regular way. All the amide nitrogen data reported in this paper, however, were obtained by PHILLIPS' original method.

SULPHUR FRACTIONS.—Since the total sulphur in the alcoholic residue was determined after drying to constant weight in the vacuum oven, it was deemed essential to determine whether the sunflower stems contained any volatile sulphur that would be lost during the drying process. Tests by the method of PLATENIUS (**25**) showed little, if any, present; so this fraction was not estimated. Total sulphur in the extract after evaporating off the alcohol was determined by the official sodium peroxide method (**1**), and in the residue by the Parr Co. bomb method essentially as described in their direction booklet. This bomb method is a great improvement over the open-crucible sodium peroxide method.

Results

Symptoms of sulphur deficiency developed quickly, appearing first in the first true leaves. In less than 2 weeks after planting, these leaves were decidedly less green than were the corresponding leaves of the plus-sulphur plants. Also at this time some of the minus-sulphur plants were not so tall as the plants supplied with a complete nutrient solution. These symptoms increased in intensity as the plants became older. At the time of harvest the leaves of the sulphur-deficient plants were yellow-green in color, smaller, and the stems much thinner than in the case of the plus-sulphur plants. No definite gradation of greenness was noticed at any time. While the minus-sulphur plants were shorter than those supplied with sulphur, yet they elongated markedly. A few plants of each group were grown to maturity. Some of the sulphur-deficient plants reached a height of almost 200 cm.; the tallest plus-sulphur plant was about 300 cm. in height.

It is evident from table 2 that the stems of the minus-sulphur plants are much smaller than those of the plus-sulphur, the latter at each level weighing more than

three times the former. In each case there is an increase in green weight from the top to the bottom of the plant. The two groups do not differ much in moisture content at each level. The percentage moisture of each group decreases from the top to the bottom of the plant, as would be expected; the percentage dry weight of course shows the reverse gradation. Tables 3–8 give the data for the chemical com-

TABLE 2

DATA FROM 100 PLANTS

Stem level	Green weight		Percentage water		Percentage dry weight	
	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S
Upper	387.7	1399.8	93.767	93.530	6.233	6.470
Middle	488.5	1769.6	93.143	93.147	6.857	6.853
Lower	628.2	2185.2	89.583	90.383	10.417	9.617

TABLE 3

NITROGEN FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

Stem level	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S
	Total N		Total organic N		70% alcohol-soluble N		70% alcohol-insoluble N	
Upper	5.526	3.824	3.188	1.717	4.537	2.656	0.989	1.168
Middle	4.673	2.968	2.431	1.112	4.145	2.221	0.528	0.747
Lower	2.397	1.801	1.231	0.766	1.971	1.249	0.426	0.552
	Ammonia N		Amino N		Amide N		Nitrate N	
Upper	0.163	0.022	0.798	0.185	0.192	0.004	2.339	2.107
Middle	0.092	0.011	0.625	0.115	0.156	0.004	2.243	1.856
Lower	0.035	0.002	0.233	0.085	0.071	0.000	1.166	1.035

position, figured on the basis of the dry weight and also calculated on the absolute basis.

NITROGEN FRACTIONS.—Table 3 shows that there is a positive gradient from top to bottom of the stems of each group of plants, but at each level the percentage is much higher for the minus-sulphur stems than for the plus-sulphur, except of course in the case of 70 per cent alcohol-insoluble nitrogen. A comparison of the two groups at the three levels shows that the sulphur-deficient stems contain seven to seventeen times as much ammonia and two to five times as much amino nitro-

gen as the plus-sulphur. Very little amide was found in the latter stems (none at the lower level), while considerable is found in the former. Nitrates accumulate to a certain extent in the minus-sulphur stems. In each group the nitrates make up a rather large proportion of the nitrogen that dissolves in 70 per cent alcohol. Most of the total nitrogen dissolves in 70 per cent alcohol; there is a rather small amount left in the residue (70 per cent alcohol-insoluble nitrogen).

Table 4 gives the data for the nitrogen fractions on an absolute basis. The gradients of the percentage data are more or less leveled out when the data are figured on an absolute basis, because the gradients of the percentages and the absolute

TABLE 4

NITROGEN FRACTIONS (GRAMS PER 100 PLANTS)

Stem level	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S
	Total N		Total organic N		70% alcohol-soluble N		70% alcohol-insoluble N	
Upper	1.335	3.463	0.770	1.555	1.096	2.405	0.239	1.057
Middle	1.565	3.599	0.814	1.385	1.388	2.693	0.176	0.905
Lower	1.568	3.784	0.805	1.609	1.290	2.624	0.278	1.160
	Ammonia N		Amino N		Amide N		Nitrate N	
Upper	0.039	0.019	0.192	0.167	0.046	0.003	0.565	1.908
Middle	0.030	0.013	0.209	0.139	0.052	0.004	0.751	2.250
Lower	0.022	0.004	0.145	0.178	0.046	0.000	0.763	2.175

weights of the stem sections are in opposite directions (table 2). Since the plus-sulphur stems weighed much more than the minus-sulphur, they are higher in the various nitrogen fractions, except ammonia, amide, and amino acids. The fact that despite the much smaller stems a larger amount of these fractions is present in the sulphur-deficient stems emphasizes their accumulation as a result of sulphur deficiency.

CARBOHYDRATE FRACTIONS.—Table 5 records the data for the carbohydrate fractions on a percentage basis. The minus-sulphur stems are somewhat higher in starch and acid-hydrolyzable carbohydrates but are much lower in reducing sugars and sucrose than the plus-sulphur. They contain less than one-fourth as much reducing sugar and less than one-third as much sucrose as the stems of the plants grown with complete nutrient. A large amount of reducing sugar is found in the plus-sulphur stems, reaching at the upper level more than 19 per cent of the dry weight. Reducing sugars make up a rather large part of the total carbohydrates of

both groups of plants; relatively small amounts of polysaccharides and sucrose are present. The percentages of total carbohydrates are not given, but it is evident that, because of the large sugar content of the plus-sulphur stems, they are to be considered high-carbohydrate in comparison with the minus-sulphur.

The gradient of reducing sugar is positive downward in each group of plants, but the data for sucrose exhibit no definite trend. While the starch gradient is positive downward, the acid-hydrolyzable gradient is in the opposite direction.

TABLE 5

CARBOHYDRATE FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

STEM LEVEL	REDUCING SUGARS		SUCROSE		STARCH		ACID-HYDROLYZABLE CARBOHYDRATES	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper	5.005	19.592	0.365	2.472	1.296	0.725	0.516	0.323
Middle	3.660	16.767	0.900	2.480	0.735	0.537	0.972	0.512
Lower	2.246	10.730	0.747	1.738	0.315	0.098	1.305	1.095

TABLE 6

CARBOHYDRATE FRACTIONS (GRAMS PER 100 PLANTS)

STEM LEVEL	REDUCING SUGARS		SUCROSE		STARCH		ACID-HYDROLYZABLE CARBOHYDRATES	
	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S	MINUS S	PLUS S
Upper	1.209	17.744	0.088	2.238	0.313	0.656	0.124	0.292
Middle	1.226	20.334	0.301	3.008	0.246	0.651	0.325	0.620
Lower	1.470	22.550	0.488	3.652	0.206	0.205	0.854	2.301

The data for carbohydrates calculated on an absolute basis are given in table 6. The much larger plus-sulphur stems contain considerably more of each compound than the minus-sulphur. As in the case of the nitrogen fractions, there is a tendency for the percentage gradients to level out when the data are figured on an absolute basis. All the carbohydrate fractions show a definite gradient, although it is not in all cases in the same direction as when the data are figured on a percentage basis. The positive gradients of reducing sugars on the latter basis become negative; sucrose which showed no gradient on a percentage basis becomes negative; starch remains positive; and the negative gradient of the acid-hydrolyzable carbohydrates is intensified.

SULPHUR FRACTIONS.—The minus-sulphur stems contain at each level considerably more of the sulphur compounds soluble in 70 per cent alcohol than the plus-

sulphur; the sulphur not soluble in the alcohol and total sulphur are higher in the latter stems (table 7). The sulphur gradients are not so regular in all cases as are the nitrogen and carbohydrate gradients previously described, but the upper levels are considerably higher in the various sulphur fractions than the lower. On an absolute basis (table 8) the large plus-sulphur stems contain much more of each of the sulphur fractions than the minus-sulphur. When the data are figured on this basis there is the same tendency for the percentage gradients to level out that was noted for the carbohydrate and nitrogen compounds.

TABLE 7

SULPHUR FRACTIONS (PERCENTAGE ON DRY-WEIGHT BASIS)

Stem level	70% alcohol-soluble S		70% alcohol-insoluble S		Total S	
	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S
Upper	0.154	0.063	0.130	0.302	0.284	0.365
Middle	0.160	0.100	0.085	0.272	0.245	0.372
Lower	0.052	0.057	0.068	0.115	0.120	0.172

TABLE 8

SULPHUR FRACTIONS (GRAMS PER 100 PLANTS)

Stem level	70% alcohol-soluble S		70% alcohol-insoluble S		Total S	
	Minus S	Plus S	Minus S	Plus S	Minus S	Plus S
Upper	0.037	0.057	0.031	0.273	0.068	0.331
Middle	0.053	0.121	0.028	0.329	0.082	0.451
Lower	0.034	0.119	0.044	0.241	0.079	0.361

Discussion

Appearance of plant: Chemical composition

The visible effects of sulphur deficiency on the sunflower are similar to those on the soybean (**5**): the yellow-green color and reduced size of the leaves and the thinner stems as compared with the plus-sulphur plants (fig. 1). Stem elongation is a prominent feature in both cases.

As Nightingale (**21**) points out, the symptoms of sulphur deficiency (especially as regards color) are similar to those caused by the deficiency of other elements (for example, nitrogen, potassium, phosphorus, and calcium), and may be explained on a similar basis, namely, poor nitrate assimilation, due in the first case of course to the lack of nitrates in the nutrient solution and in the other deficiencies to the

lowered reducase content of the plant (7). Nitrates are not assimilated properly and the result is only slight chlorophyll development.

The effects of sulphur deficiency on the chemical composition of the sunflower stems were also similar to those of the soybean (5). The soluble organic nitrogen compounds—ammonia, amide, and amino acids—were much higher in the minus-sulphur stems than in the plus (table 3). The soluble sulphur fractions also accumulated in the sulphur-deficient stems (table 7). These fractions were not determined

FIG. 1.—Sunflower plants: plus-sulphur and minus-sulphur

in the case of the soybean. Nitrates and the carbohydrates—starch and acid-hydrolyzable carbohydrates—also were higher in the sulphur-deficient stems, but the sugars were lower (tables 3, 5). Nitrates, starch, and acid-hydrolyzable carbohydrates no doubt accumulated in the minus-sulphur stems, due to the fact that protein synthesis for which these compounds are needed proceeded slowly because of the low reducase content of the plants (7).

The carbohydrate situation resulting from sulphur deficiency varies in different plants. All forms of carbohydrates accumulate in a minus-sulphur tomato stem (21). While a sulphur-deficient soybean stem is lower in sugars than a plus-sulphur stem, it may be enough higher in polysaccharides to be high-carbohydrate when all forms of carbohydrates are included (5). On the other hand, although the minus-sulphur sunflower stems are slightly higher in starch and acid-hydrolyzable

carbohydrates, they are so much lower in sugars than are the stems of the plants grown with complete nutrient that they are to be considered low-carbohydrate. As already stated, the accumulation of carbohydrates, and also nitrates, in sulphur-deficient plants is to be accounted for by the low protein synthesis resulting from their low reducase content. It is not known why reduced protein synthesis causes the accumulation in the tomato of all forms of carbohydrates, but in the soybean and sunflower only of the polysaccharides.

Reutilization

It was stated at the beginning of this paper that minus-sulphur plants through proteolysis seem to secure organic nitrogen and sulphur compounds for use in stem elongation. This section discusses various lines of evidence for this theory of reutilization, and refers to another process that may account partly for the accumulation of soluble organic nitrogen and sulphur compounds in a sulphur-deficient plant.

Gradients.—As regards deficiencies other than sulphur, certain work indicates transfer of materials from the older parts of the plant to the younger. For example, the investigations on barley by Richards and Templeman (**26**) indicate that deficiency of phosphorus, potassium, or nitrogen resulted in the transfer of the respective element from the old leaves to the meristem. Mason and Phyllis (**15**) showed that during nitrogen deficiency the younger leaves of cotton increased their nitrogen content at the expense of the older leaves at the base of the plant. Also, carbohydrates and organic nitrogen compounds seem to be translocated on a gradient basis; that is, from a region of greater concentration to one of less. This has been more especially demonstrated for downward transport of carbohydrates (**16**).

In the present work the sunflower stems were analyzed at three levels with the idea that the gradient data might give some indication of whether there was marked proteolysis in the lower part of the minus-sulphur stem and upward transport of the resulting compounds. If these processes are going on rapidly, it might be expected that the gradients of the nitrogen and sulphur compounds would be positive upward. But as shown by tables 3 and 7, the gradients give no support to this theory of upward transport. The gradients of all the nitrogenous and sulphur compounds are negative upward rather than positive upward, although the gradients of the latter compounds are not so regular as the former.

For a number of reasons, however, the nature of the gradients should not be overemphasized as evidence against upward transport of the compounds just mentioned. One reason is that, although organic nitrogen compounds seem to be translocated on a gradient basis, it may not be easy to prove this, as Maskell and Mason (**16**) found. Although their work had shown that carbohydrates were

translocated down the cotton stem in the bark along a marked positive gradient, and although their preliminary work (13) indicated that nitrogen was also transported downward in the bark on a gradient basis, determination of the gradients (14) showed that nitrogen was moving downward against a gradient of organic crystalloid nitrogen. It was suggested that the gradient of the mobile form of nitrogen was positive downward but that this was masked in some way, perhaps by a greater gradient in the reverse direction of static storage nitrogen, so that the net gradient was negative downward. Later work (15) gave support to this masking effect of storage nitrogen. Perhaps a similar masking effect operates in the present work to cause the gradient to be negative upward rather than positive upward.

Another reason for not necessarily regarding the gradients as evidence against upward transport of nitrogenous and sulphur compounds is that my analyses are rather limited. Only one analysis was made and that rather late in the vegetative period. The stem sections were analyzed as a whole; they were not divided into different tissues. Also no ringing experiments were performed.

It is possible that earlier in the vegetative period the gradients were positive upward, but that owing to transport of nitrogenous and sulphur compounds from the base of the plant to the top they became negative, as MASON and PHYLLIS (15) found for nitrogen in cotton stems. If this were true in the sunflower, analyses at intervals would have demonstrated it. Since most of the evidence indicates that organic compounds are translocated in the phloem, the bark should have been analyzed separately from the other tissues. The gradient in the bark might have been masked by a greater reverse gradient in the other tissues. If there is upward transport of nitrogen and sulphur, then a ring near the top would cause an accumulation of these compounds below the ring. In the case of the cotton stem, MASKELL and MASON (13) found that nitrogenous compounds accumulated above a ring, indicating downward transfer. They determined the gradients in bark and wood separately (14).

After full consideration of the preceding points, however, it must be admitted that the gradient data do not support the theory of proteolysis in the lower parts of the minus-sulphur stem and transfer of the resulting material to the top to be used in stem elongation. Further work is needed, including ringing experiments, before a definite conclusion can be made.

Of course, since the gradients of the sulphur-deficient stems are positive downward, they might be interpreted as indicating synthesis in the upper parts of the minus-sulphur plants and transfer downward of the organic nitrogen and sulphur compounds. But synthesis is no doubt at a minimum in these plants because of the probably low reducase content and lack of sulphur. This interpretation would not seem to apply, therefore, although in the absence of ringing experiments no positive statement can be made. And since the gradients are in the same direction in

the plus-sulphur stems where synthesis is going on rapidly, as in the minus-sulphur stems where it is not, the gradients of the former should also probably not be interpreted as indicating downward transfer.

The gradients of nitrogenous and sulphur compounds are in the same direction as that of metabolically active cells; both are positive downward. It would seem that the positive gradients of these compounds merely result from the metabolic processes of these cells, the compounds composing the gradients being used in or resulting from these processes. This was ENGARD's (9) interpretation of the positive gradients of nitrogenous compounds in the vegetative canes of the raspberry growing in soil. Nitrates are distributed freely in the canes; they are available in all living cells for the synthesis of organic, nitrogenous compounds. Since the number of living, protoplasm-containing cells is greater in the upper parts of the canes than in the lower, synthesis goes on more rapidly there; hence the positive gradients. That the direction of the gradients did not indicate translocation downward was indicated by the failure of a ring to reverse the gradient or even to make it level. A similar explanation would seem to apply to the sunflower. As shown by table 3, the nitrate gradient is positive downward. This is the same as the gradient of living cells, so nitrates are no doubt available for synthesis of organic nitrogenous compounds in these cells. And synthesis probably accounts mainly for the positive gradients of the plus-sulphur stems. But in the case of the minus-sulphur stems, since synthesis is probably at a minimum because of low reducase content and lack of sulphur, proteolysis rather than synthesis is probably more important in accounting for the gradients of the soluble organic nitrogenous and sulphur compounds. If there is little upward transfer of the materials resulting from the proteolysis, then the extent of proteolysis itself is represented by a gradient which is positive downward; in other words, the proteolysis may go on mainly in the place where resulting compounds are found, with perhaps transfer through only short distances. LEONARD (10), in a study of field-grown sunflower plants, also found positive downward gradients of nitrogenous compounds in the stems. His data indicate that synthesis of these compounds took place mainly in the young leaves, the upper pith, and the head.

This section is not primarily concerned with the carbohydrates. It is interesting, however, that there is the same correlation between reducing sugars and starch (table 5) and protoplasm-containing cells already noted for the nitrogenous and sulphur compounds; the gradients of these carbohydrates and of the living cells are both positive downward. And perhaps a similar explanation may be given for this correlation as was given in the preceding paragraph in the case of the nitrogen and sulphur compounds. ENGARD (8) found that all the carbohydrate gradients (with the possible exception of sucrose) in the raspberry canes were positive downward and explained these mainly on the basis of temporary storage in living cells,

or in the case of the polysaccharides, cells associated with living cells, although part of the gradient of reducing sugars was translocatory, as shown by ringing experiments. This explanation probably holds for the reducing sugars and starch of the present experiment. As shown by table 5, while the starch gradient is positive downward the acid-hydrolyzable gradient is negative downward. Sucrose exhibits no gradients.

ACCUMULATION OF AMIDE NITROGEN.—The gradients are hard to interpret and cannot be used as definite evidence of proteolysis and reutilization. But that proteolysis is going on, especially in the minus-sulphur stems, is indicated by the accumulation in these stems of the soluble organic-nitrogen fractions of which amides form an abnormal percentage. As shown by table 3, the fractions ammonia, amino acids, and amides are several times higher in the minus-sulphur stems than the plus-sulphur, and amides make up a much greater proportion of the soluble nitrogen of the former stems than of the latter. Only a trace of amide was found in the plus-sulphur stems. Table 7 shows that soluble sulphur is also considerably higher in the minus-sulphur stems. The soluble sulphur fraction includes not only organic sulphur but also any sulphate sulphur that may be present. It would have been interesting to have fractioned the soluble sulphur into sulphate sulphur and organic sulphur. NIGHTINGALE (**21**) got indications of the proteolytic formation of sulphates in germinating seeds, and WOOD and BARRIEN (**37**) report that under conditions of starvation in the dark protein sulphur decreases and is accompanied by an equal increase in sulphate sulphur.

The preceding situation, at least as regards the nitrogen fractions, usually prevails when proteolysis is taking place in plants. The germination of seeds affords a familiar illustration. During germination the carbohydrates decrease greatly, the proteins are hydrolyzed to amino acids and amides, part of the amino acids is oxidized (ammonia being produced), and the ammonia is combined with carbon compounds arising from this oxidation of the amino acids (or from carbohydrates) to give amides. While part of the amide pre-existed in the seed proteins, most of it is of secondary origin, as just described; there is much more amide in the seedling than was found in the proteins of the seed. This secondary production of amide is at present regarded as part of the respiratory process. Usually it does not go on rapidly in the plant until the carbohydrates have become too deficient to supply the necessary energy for the plant's life processes. In this situation energy is obtained by the oxidation of amino acids. CHIBNALL (**2**), VICKERY (**34**), NIGHTINGALE (**22**), and MURNEEK (**18**) discuss proteolysis and amide metabolism.

Proteolysis in plants is a common phenomenon. Besides the germination of seeds, it has been studied especially in detached leaves (**2, 33, 34**) and in plants in the dark (**19**). In discussions of proteolysis, however, mineral deficiencies usually are not included. Yet on theoretical grounds it might perhaps be expected that

when an element is deficient, proteolysis would occur. As RICHARDS and TEMPLEMAN (26) point out, it does not seem likely that a deficient element would be found in the plant in a form not utilizable in metabolism; that is, it might be expected that when the supply of an element is deficient organic compounds of this element would be broken down, releasing the element in a form that could be used in metabolism. And, as previously mentioned, the work of these investigators on nitrogen, phosphorus, and potassium deficiencies indicated transfer of these elements from the old leaves of the barley to the meristem. Also, analyses of the nitrogen-deficient cotton plant showed transfer of nitrogen from the old leaves to the young (15).

Although doubtless mineral deficiency commonly causes proteolysis, the process does not seem rapid, at least in the case of the deficiencies being considered, unless the plant is put in the dark or there is injury to the protoplasm, as in advanced stages of phosphorus or potassium deficiency (21). In certain other deficiencies (for example, calcium and boron) proteolysis and reutilization may be even slower. NIGHTINGALE (20) found that the root and stem apices of calcium-deficient tomato plants died while there was still much calcium in the forms of calcium oxalate crystals and combined calcium in the older tissues. Boron deficiency also affects the meristematic tissues of the apices of the plant. The fact that these deficiencies, including nitrogen, do not normally result in rapid proteolysis may be at least partly conditioned by the fact that they result in the accumulation of carbohydrates. High carbohydrate content favors synthesis rather than hydrolysis of proteins.

At first sight at least, proteolysis in sulphur-deficient plants seems to constitute a rather special case. It is usually correlated with a reduction of carbohydrates. Sulphur deficiency does not seem to injure the protoplasm (21). Yet hydrolysis of the proteins goes on rapidly in a minus-sulphur plant while it is in the light and even though it is high in carbohydrates. Thus there are provided organic sulphur and nitrogen compounds which would otherwise be deficient because of the lack of sulphur in the nutrient solution and the effect of sulphur deficiency in causing a low reducase content of the plant. And the sulphur-deficient plant elongates rapidly in contrast with the other deficiencies just mentioned.

Although proteolysis in a sulphur-deficient plant seems to be abnormal, in that the process goes on rapidly even though the plant is high in carbohydrates, yet sulphur-deficient plants vary greatly as to the carbohydrate situation. All forms of carbohydrates accumulate in a minus-sulphur tomato plant (21). The soybean is decidedly lower in sugars but may be enough higher in polysaccharides to be considered high-carbohydrate in comparison with the plus-sulphur plant (5). While the sunflower is slightly higher in polysaccharides, it is so low in sugars that it should be regarded as low-carbohydrate (table 5). So proteolysis in the soybean

and the sunflower, and especially in the latter, may be considered more in line with that usually going on in plants than is true of the tomato. Proteolysis in the soybean and sunflower is associated with a reduction in the more available carbohydrates, reducing sugars and sucrose; in the tomato all forms of carbohydrates accumulate. But considering all three plants together, it would seem that the concentration of carbohydrates is not the factor controlling proteolysis.

A similar statement may be made in regard to other more commonly studied cases of proteolysis; for example, detached leaves. It is not known definitely what regulatory mechanism controls synthesis and hydrolysis of proteins. Usually high carbohydrate content favors synthesis, low carbohydrate content, hydrolysis; the "sparing action" of carbohydrates is a well-known phenomenon. But this is not always the case. Rapid proteolysis may go on under conditions which maintain a rather high level of carbohydrate or even cause a decided increase in the concentration; as for example, in the case of some of the experiments with detached leaves in the light or in glucose solutions in the dark (**2, 33, 34**). So the carbohydrate content does not give an adequate explanation. As CHIBNALL (**2**) points out, "We do not yet understand the reason why, when certain leaves are detached from the plant, protein decomposition can be detected within a few hours." CHIBNALL gives an excellent discussion of the various theories regarding regulation of protein metabolism in leaves. A similar statement may perhaps be made in regard to sulphur-deficient plants; it is not known why, when sulphur is deficient, proteolysis occurs. But some factor other than the carbohydrate concentration would seem to be controlling the process.

It would seem, therefore, that proteolysis in sulphur-deficient plants should not after all be considered so different from other cases of proteolysis. Proteolysis may go on in sulphur-deficient plants of widely varying carbohydrate concentration; the same is true of other more generally discussed types of proteolysis.

NEED OF SULPHUR FOR PROTEIN SYNTHESIS.—Calculations reported in previous work on the soybean (**5**) indicate that the need of sulphur for the synthesis of proteins from amino acids and amides accounts partly for the accumulation of soluble organic nitrogen compounds in a sulphur-deficient plant. The calculations involved comparing the organic nitrogen content of seed and plant. Similar calculations in the case of the sunflower lead to the same conclusion. In the present work the seed was not analyzed, but seed from the same lot has been used in class experiments in the laboratory. The seed contains 4.5 per cent total nitrogen.[1] The average weight of a seed is 62 mg. Assuming no nitrate present, each seed contains 2.79 mg. of total organic nitrogen. Table 4 records the data of the nitrogen fractions on an absolute basis for each level. If the organic nitrogen for the entire stem is determined, it will be found that each stem, not counting leaves and roots,

[1] Miss DAPHNE SWARTZ kindly supplied the determinations of total nitrogen and weight of seed.

contains more than eight times as much total organic nitrogen as the seed from which the plant developed. There must have been synthesis of much organic nitrogenous compounds in the sulphur-deficient stems. There was, of course, synthesis of much more of these compounds in the plus-sulphur plant, as shown by the table.

Synthesis of amino acids is reduced in a sulphur-deficient plant because of low reducase content, as has already been mentioned. Sulphur deficiency does not, however, interfere with reduction of nitrates as much as other deficiencies (7), so no doubt there is a constant, slow synthesis of amino acids. But sulphur-containing amino acids cannot be synthesized because of the lack of sulphur in the nutrient solution. Since nearly all plant proteins contain sulphur, the nonsulphur-containing amino acids presumably cannot be condensed to proteins; hence they accumulate in a minus-sulphur plant. In line with this viewpoint is NIGHTINGALE'S (21) observation that very little of the soluble organic sulphur of the sulphur-deficient tomato plants was cysteine or glutathione. It would seem, therefore, that part of the accumulation of soluble organic nitrogenous compounds in a minus-sulphur plant is due to the need of sulphur for the synthesis of proteins from amino acids and amides.

SUMMARY OF REUTILIZATION.—Proteolysis seems to proceed readily in a minus-sulphur plant, even though it is high in carbohydrates. This is indicated mainly by the accumulation of soluble organic sulphur and nitrogenous compounds, an abnormal percentage of the nitrogenous compounds being amides. Thus there are provided these compounds under conditions which would otherwise cause them to be quite deficient, that is, low nitrate assimilation and the lack of sulphur in the nutrient solution. As a result, stem elongation occurs rapidly. Although the gradient data are limited, the indications are that the proteolysis proceeds mainly in or near the place where the soluble compounds are found, with perhaps transfer through only short distances. There may be no transport of materials from the base to the tip of the plant. Part of the accumulation of the soluble nitrogenous compounds is probably due to the need of sulphur-containing amino acids for the condensation of amino acids to proteins.

IMPORTANCE OF ACID-HYDROLYZABLE CARBOHYDRATES IN METABOLISM

This work has not been concerned primarily with a study of the importance of the acid-hydrolyzable carbohydrates in the metabolism of the sunflower. However, a consideration of the carbohydrate data (table 5) shows that, while the starch and reducing sugar gradients are in the same direction as the metabolically active cells, the gradient of the acid-hydrolyzable substances is in the reverse direction and corresponds with the gradient of nonliving cells. Since starch is always regarded as readily usable by the plant, the fact that the acid-hydrolyzable gradi-

ent is in the reverse direction might be regarded as indicating that under the conditions of this experiment the acid-hydrolyzable carbohydrates of the sunflower are not usable, or at least not so much so as starch. Since only one analysis was made, however, no definite conclusions can be drawn.

It is a moot question whether the acid-hydrolyzable carbohydrates are used in metabolism or whether they should be considered as rather permanent structural constituents of the cell wall. CLEMENTS (3) is inclined to regard these compounds as readily usable by the plant. On the other hand, WINKLER and WILLIAMS (36), after a critical review of the literature, conclude that—except in the case of the seeds of a few plants—there is no definite evidence that these substances function as reserve materials. NIGHTINGALE (19) takes the somewhat intermediate viewpoint that these carbohydrates are probably not used by the plant until starch, dextrin, and sugars have become almost depleted.

The acid-hydrolyzable carbohydrates constitute a very heterogeneous group of carbohydrates, whose importance in metabolism varies, depending on a number of factors. The nature of these carbohydrates differs in different plants and in the same plant in different tissues and under diverse climatic conditions. Also their usability is probably affected by other more readily available carbohydrates. CLEMENTS (3) concluded that under favorable conditions of growth the acid-hydrolyzable carbohydrates of the leaves of the sunflower, potato, and soybean were serving as important food reserves, since they fluctuated widely during the day; lack of fluctuation under drought conditions indicated that they were not being used in metabolism. LEONARD (10) found much greater variation in the acid-hydrolyzable material of the bark of field-grown sunflowers during the season than of the wood, indicating greater usability in the former case. ENGARD (8) states that the function of acid-hydrolyzable substances "in metabolism is variable in plants and depends to a great extent upon the kinds and amounts of other more readily utilizable carbohydrates." He found very little starch in the raspberry canes, and his work indicated that the acid-hydrolyzable substances were more important than starch in the metabolism of the raspberry. These substances were removed in two fractions, one by 3 hours' hydrolysis, the other by 12 hours' further hydrolysis. The first was regarded as more important in metabolism. The sunflower plants of this experiment also contained little starch (table 5), yet there are indications that the starch is more related to metabolism than are the acid-hydrolyzable substances. Analyses should have been made at intervals, however, to compare the fluctuations in the amounts of starch and the acid-hydrolyzable material.

It would seem impossible to make any categorical statement as to the importance of the acid-hydrolyzable carbohydrates in metabolism. Sometimes they are important, sometimes they are not. Their usability varies with the kind of

plant, carbohydrate, tissue, and climatic conditions, with the amounts of other carbohydrates, and perhaps other factors. Data indicating lack of importance in metabolism should not be overemphasized. As NIGHTINGALE (19) points out, it is hardly fair to use data obtained by acid hydrolysis as a criterion of what the enzymes of the plant can do. And finally, as CLEMENT'S (3) work indicates, even if the acid-hydrolyzable carbohydrates are not important in metabolism, they may have other important functions, such as tissue strengthening and protection from drought.

Summary

1. The visible effects of sulphur deficiency in the sunflower were similar to those noted in previous work on the soybean: the yellow-green color of the leaves, the smaller leaves, and the thinner stems as compared with the plus-sulphur plants. Stem elongation was a prominent feature.

2. The soluble organic nitrogen fractions and nitrates accumulated in the minus-sulphur stems as compared with the plus-sulphur. This was also true of the soybean. Soluble sulphur compounds were also higher in the sulphur-deficient sunflower stems. These were not determined in the case of the soybean.

3. While the minus-sulphur sunflower stems were somewhat higher in starch and acid-hydrolyzable carbohydrates than the plus-sulphur, they were so much lower in sugars that they were to be considered low-carbohydrate stems. On the other hand, although the sulphur-deficient soybean stems were lower in sugar than the plus-sulphur, they were so much higher in the polysaccharides that they were to be regarded as high carbohydrate. All forms of carbohydrates accumulate in a minus-sulphur tomato stem. So plants vary in this regard.

4. The accumulation of nitrates and carbohydrates in minus-sulphur plants seems to be due to low nitrate assimilation, which results from the low reducase content of the plants. The low nitrate assimilation also no doubt accounts for the yellow color of the sulphur-deficient leaves, as is true of other deficiencies; for example, potassium, calcium, and phosphorus. Accumulation of soluble organic nitrogen fractions and soluble sulphur compounds seems to be due mainly to proteolysis. Proteolysis and reutilization seem to be more prominent features of sulphur deficiency than is true of most other deficiencies.

5. Part of the accumulation of the soluble organic nitrogen fraction as a result of sulphur deficiency may be due to the lack of sulphur-containing amino acids for the completion of protein synthesis.

6. It is not known why low protein synthesis results in the accumulation in the tomato of all forms of carbohydrates but in the soybean and sunflower of only starch and acid-hydrolyzable carbohydrates.

7. Since the gradients of the nitrogen and sulphur fractions are negative upward rather than positive upward, they offer no support for the theory of proteolysis

in the lower levels of the sulphur-deficient stems and transport of the resulting materials to the tips to be used in stem elongation.

8. Since only one analysis was made (and that rather late in the vegetative period), the different tissues were not analyzed separately, and no ringing experiments were performed, perhaps the nature of the gradients should not necessarily be regarded as evidence against upward transport.

9. It may be, however, that proteolysis in the minus-sulphur stems goes on at or near the place where the resulting materials are found, with transfer at the most through only short distances.

10. The gradients of nitrogenous and sulphur compounds were in the same direction as that of the metabolically active cells, namely, positive downward. It seems likely that the gradients result from the metabolic activities of these cells: proteolysis in the case of the sulphur-deficient stems, synthesis in the case of the plus-sulphur.

11. The positive downward gradients of starch and reducing sugars may also result from synthesis in living cells, although in this case the gradients may be partly translocatory.

12. The gradients in themselves do not afford evidence for the theory of proteolysis and reutilization. But the accumulation in the minus-sulphur stems of soluble organic nitrogen fractions, of which amide forms an abnormal percentage, indicates that rapid proteolysis is going on in these stems. This situation usually prevails in proteolysis.

13. Proteolysis in a sulphur-deficient plant seems abnormal in that it proceeds even though the plant is high in carbohydrates. Usually proteolysis is associated with a reduction in carbohydrates. But it would seem that carbohydrate content is not the controlling factor in proteolysis. The process may go on in detached leaves, for example, under conditions of either high or low carbohydrate content. The same may be said for minus-sulphur plants. The sulphur-deficient tomato and soybean stems may be regarded as high carbohydrate, although sulphur deficiency results in a lower sugar content of the soybean stems. On the other hand, a minus-sulphur sunflower stem is to be considered low in carbohydrates although slightly higher than the plus-sulphur in starch and acid-hydrolyzable carbohydrates. From this standpoint proteolysis resulting from sulphur deficiency should not be considered so abnormal.

14. While the starch and reducing sugar gradients of the sunflower stem are in the direction of the metabolically active cells, positive downward, the acid-hydrolyzable gradient is in the reverse direction, that of the nonliving cells. This may be regarded as evidence that under the conditions of this experiment the acid-hydrolyzable carbohydrates are not so important in metabolism as starch and reducing sugars.

15. No categorical statement can be made as to the importance of the acid-hydrolyzable carbohydrates in metabolism of plants. Their usability may vary with the kind of plant, carbohydrate, climatic conditions during growth, with the amount of other more available carbohydrates present, and perhaps other factors.

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EFFECTS OF SOME ENVIRONMENTAL FACTORS ON PHOTOPERIODIC INDUCTION OF BEET AND DILL[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 521

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(WITH SIX FIGURES)

Introduction

Among other factors of the environment, temperature and photoperiod are important in influencing the disposition of many plants to differentiate floral primordia. This is particularly true for plants of a biennial habit. A number of investigators (**2, 8, 14, 18, 19, 20**) agree that the promotive effects of low temperature in initiating seed-stalk development may be subsequently decreased at higher temperatures. Inasmuch as the temperature phases of many photoperiodically sensitive plants may be very short and yet effective in influencing subsequent response to light, as is well illustrated by vernalization experiments, care must be exercised in interpreting results in terms either of light reaction or of reaction to temperature.

If an interpretation is to be made in terms of photoperiodic reaction alone, it is necessary to select plants which do not require a temperature phase in order to render them sensitive to the proper photoperiod for flowering. Since some of the work reported here was performed to determine whether the theories of floral initiation which have been advanced (**4, 12, 5**) can be used to interpret the flowering response of long-day plants, the plants used were not temperature limited.

Dill (*Anethum graveolens* Linn.) was previously reported (**7**) to be a sensitive long-day plant having a short induction period and the ability to produce quickly a flowering stalk after four long photoperiods when returned to short photoperiod conditions. MURNEEK (**13**) has recently emphasized the sensitivity of dill, and in addition has pointed out that it, like Biloxi soybean, will flower if kept for sufficient time on photoperiods not considered conducive to flowering. Previous work had also shown that dill did not require a low temperature treatment in order to be photoperiodically sensitive.

The variety of beet (*Beta vulgaris* L.) chosen differs from most varieties in that it does not have a definite temperature phase. There has been very little modifica-

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tion in its flowering response over rather wide temperature ranges in the greenhouse. Another characteristic of this variety is its capacity to flower vigorously after fourteen or more long photoperiods (19–21 hours of light and 5–3 hours of darkness) when returned to short photoperiod (8 hours of light out of 24).

The dill seeds were obtained from commercial sources; the beet seeds were obtained from Dr. E. Carsner, Riverside, California.

General methods

No attempt was made to regulate either the temperature or the humidity of the greenhouse, other than to prevent excessively high or low temperatures. The rooms were heated whenever the temperature fell below 18° C. and ventilated when temperatures rose above 32° C. Humidity was partially controlled by spraying the walks and walls with tap water. The plants were watered twice daily with tap water and at intervals with a modified SHIVE's (**16**) R_2S_5 nutrient solution.

The seeds were germinated in flats containing a light garden soil. After planting the seeds, the flats were immediately placed either on benches or trucks and subjected to cycles of the desired photoperiod. The beets were usually germinated on shallow greenhouse benches of the usual type, except that they had low rectangular wooden frameworks arranged over them. These frames supported a double thickness of black sateen cloth which was placed over them nightly to control the length of the photoperiod. The dill seeds were germinated on small trucks which were rolled into light-tight sheds each evening.

In the course of the work with dill, it was found necessary to transplant the very young seedlings from flats to $3\frac{1}{2}$-inch pots. If the leaves had attained a length of 4–5 inches and there were more than three or four expanded leaves present at the time of transplanting they were considered unreliable test plants, since they tended to flower spontaneously even though maintained on cycles of short photoperiod. The cause of this tendency to flower has not been investigated critically, but there is some evidence that injury to leaves and roots during the transplanting process may in some way alter the metabolism of the plant sufficiently to bring about flowering. Further evidence to support this viewpoint is given later. All dill plants were maintained on a 9-hour photoperiod until they were used experimentally.

The beet seedlings were transplanted singly to $3\frac{1}{2}$-inch pots when their leaves were 4–6 inches long. After transplanting they were returned to the short photoperiod benches. These benches were covered each evening at 4:00 P.M. with the black sateen cloths and were uncovered the following morning at 8:00 A.M., thus affording 16 hours of darkness and 8 hours of light.

The response considered of primary importance in both plants was the change from the rosette condition of the main axis to elongation of the stem or seed stalk.

Elongation of the stem was not always accompanied by production of normal, fertile flowers. In some instances beet stems would be slightly elongated but fail to produce flowers, or if flowers were formed they were reduced in size and sterile. This type of response was usually the result of what was considered incomplete induction. In all the experiments with dill, rapidity of response was measured in terms of the average length of the stems at the time of harvest. Dill plants were usually harvested in any given experiment when the inflorescences of the plants receiving any one treatment began to unfold.

Investigation

Experiments with dill

The experiments were planned to test the effects on vegetative growth and reproductive response of (*a*) continuous illumination of varying intensities from time of germination; (*b*) continuous illumination of relatively constant intensity from time of germination; (*c*) interrupting the dark portion of the cycle with light for different lengths of time; (*d*) high light intensity while on short photoperiod, followed by high intensity on long photoperiod or by low intensity on continuous photoperiod; (*e*) low light intensity followed by intensities varying from 50 to 1000 foot-candles; (*f*) different intensities of red light on number of days necessary for induction; (*g*) low temperature during induction period on flowering response. Another experiment was performed in order to determine the effect of certain types of injury on subsequent development of dill.

Continuous illumination of varying intensities from time of germination.—To determine whether dill will germinate and grow to maturity under conditions of continuous illumination of high intensity, seeds were germinated in the greenhouse April 18, 1940. These seeds were scattered on the surface of the soil, which was kept moist by frequently sprinkling with a fine spray. Natural daylight was supplied from 8:00 A.M. to 5:00 P.M., at which time a reflector containing six 36″ Mazda fluorescent lamps of the daylight type was lowered into position over the seeds. The lamps, affording approximately 750 foot-candles at the soil surface, were used until the following morning at 8:00 A.M., when they were removed.

Germination began approximately 1 week after planting. Instead of the typical rosette type of growth, the stems elongated but grew only slightly in diameter. Approximately 40 days later the plants had attained a height of about 10 cm. and began unfolding their inflorescences. Since the light intensity during the first half of the experiment was more than 700 foot-candles and never fell below 200 foot-candles, it is evident that neither darkness nor light of very low intensity is necessary for the flowering response of dill.

Constant illumination at relatively constant intensities from time of germination.—This experiment was set up in a darkroom where the sole source

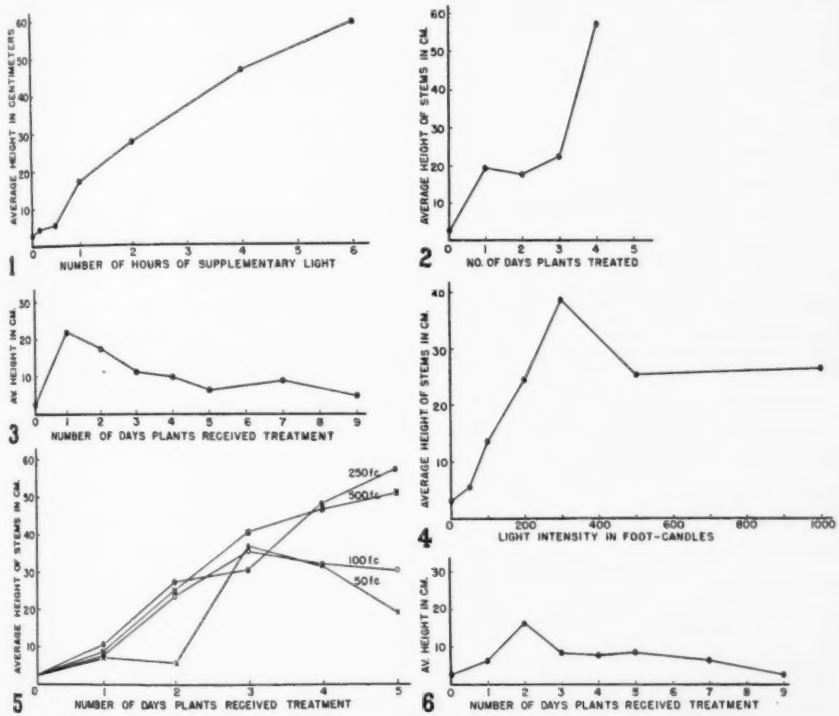
of illumination was from Mazda lamps of the daylight fluorescent type. One lot of seeds was planted at the surface of the soil, where light intensity was adjusted to approximately 1000 foot-candles. Another lot was planted similarly except that the light intensity was adjusted to approximately 500 foot-candles. The lamps burned constantly during the experiment. There was, however, a gradual increase in intensity at the surface of some of the leaves as the plants grew, since the reflectors were never readjusted. The highest intensity registered under the first reflector was approximately 1400 foot-candles and under the second, 750 foot-candles.

The plants germinated under the highest intensity grew rapidly and were sturdy. For about 30 days they remained as rosettes. After 40 days, stems of a few were elongating. Thereafter many others began to respond. Inflorescences appeared when the plants were 15–20 cm. tall. In contrast, the plants germinated under the lower light intensity grew slowly, were more succulent, and were easily bruised. They remained in the rosette stage for approximately 2 months. Several plants then began to elongate and continued to until the conclusion of the experiment September 10, when sixty-eight plants had elongated to some extent. Some stems attained a length of 50 cm. before inflorescences appeared but were bent over because of weakness.

INTERRUPTING DARK PHASE OF CYCLE WITH LIGHT FOR DIFFERENT LENGTHS OF TIME.—It has been reported that dill requires at least 14 hours of light per day for about 4 days to result in stem elongation. Inasmuch as some short-day plants react as though on long photoperiod when they are given short cycles of light during the dark period (**5, 17, 10**), tests were made to determine the response when the dark period was interrupted by an interval of light. Three hundred uniform dill plants $2\frac{1}{2}$ months old were distributed approximately equally in seven small framework compartments. All the plants received daylight 9 hours out of each 24. The plants were then darkened and supplied in six of the compartments with Mazda filament light of approximately 100 foot-candles at the leaf surface, the amount of light being automatically controlled by time clocks. Six different photoperiods were given in the middle of the dark period, ranging from 10 minutes to 6 hours. The plants in one compartment remained in darkness and served as controls. Treatment was continued for 40 days, at which time the plants receiving 6 hours of light as an interruption of the dark period were apparently at their maximum height and were beginning to shed pollen. Measurements of the length of stems of all the experimental plants were then made and averages taken (fig. 1).

Groups of plants having the dark period interrupted for either 10 minutes or 30 minutes elongated no more than the controls. In contrast, those plants receiving 1 hour of supplemental illumination (that is, a total of 10 hours of light out of each 24) elongated several centimeters during the treatment. This point is of particular

interest since the total amount of light during these 10 hours was 4 hours less than is required for stem elongation if the photoperiod is given uninterruptedly. With increasing duration of illumination (fig. 1) during the dark period up to 6 hours, the average height of the plants was almost directly proportional to the amount of supplementary illumination received.



FIGS. 1–6.—Dill: Fig. 1, effect on stem elongation of supplementary light of approximately 80 foot-candles given during middle of dark period (9 hours of daylight each day; measurements taken 40 days later). Fig. 2, response of plants receiving indicated number of cycles, each consisting of 20 hours of light and 4 of darkness (measurements taken 45 days later). Fig. 3, response of plants receiving continuous illumination of 4 foot-candles (measurements taken 45 days later). Fig. 4, effect of continuous light, given after plants had been subjected to 3 short days of low light (measurements taken 51 days later). Fig. 5, effect of red light of various intensities (measurements taken 60 days later). Fig. 6, response of plants receiving cycles, each consisting of 20 hours of light and 4 hours of dark at temperature 4.5° C. (measurements taken 36 days later).

HIGH INTENSITY PREVIOUS TO INDUCTION FOLLOWED BY HIGH INTENSITY DURING INDUCTION.—Under light conditions prevailing in January and February at Chicago, dill has an induction period of 4 days (**7**). Since light intensity plays an im-

portant role in floral initiation (**1, 5**), an experiment was performed to determine whether the average higher light intensity of June would appreciably alter the number of days required for induction. On June 15, 1939, fifty-two plants were placed on long photoperiod, while thirteen plants remained on short photoperiod as controls. The long photoperiod, consisting of 20–21 hours of light and 4–3 hours of darkness, was obtained by supplementing the ordinary day length with 80–100 foot-candles from Mazda filament lamps until 2:00 A.M. On each day for 4 days, lots of thirteen plants were transferred from the long photoperiod to conditions of short photoperiod, and the stems measured 44 days after the start of the experiment. Average heights of each group are given in figure 2.

The average height of those plants receiving one, two, and three long photoinductive cycles was about 20 cm., whereas the average height of those receiving four was approximately 60 cm., about three times the average height of the plants having fewer long photoperiods.

Since plants of which the axes begin to elongate almost invariably flower, it is evident that dill can be induced to flower during periods of high light intensity, if given only one long photoperiod. Speed of elongation and production of inflorescences are much less rapid, and total height attained at the time of flowering is usually less, than in plants receiving four long photoperiods.

HIGH LIGHT INTENSITY PREVIOUS TO INDUCTION FOLLOWED BY LOW INTENSITY DURING INDUCTION.—To test further the effect of light intensity and the duration of light in relation to the rosette and non-rosette types of growth, the following experiment was performed. On June 15, 1939, 104 plants which had been grown on short photoperiod were selected and divided into eight groups of thirteen each. Of these, the plants composing seven groups were transferred at 5:00 P.M. to a room where the light intensity (derived from a Mazda filament source) was 4 foot-candles at the surface of the leaves and was maintained at a practically constant temperature of 26° C. The thirteen plants of the remaining group were maintained on short photoperiod as a control. One group of plants receiving continuous illumination was returned to the short photoperiod trucks each day at 5:00 P.M. for the first 5 days, another group was returned on the seventh day, and the final group was returned on the ninth day. Thirty-eight days after the last transfer was made the plants were harvested and measurements of the flower stalks made (fig. 3).

Plants receiving only 1 day of continuous illumination of low intensity grew most rapidly after being replaced on short photoperiod and attained the greatest height; those treated 2 days grew a little less rapidly; and those treated 3 days grew still more slowly. The groups receiving a greater number of days of treatment differed among themselves so little in average heights that no significance can be attached to treatment longer than four 24-hour periods.

Since light intensity previous to treatment was high, one explanation for the growth effects following the first 24 hours of continuous illumination is that the threshold value for partial induction was reached with the addition of this light, even though it was of low intensity. These plants, of course, received a high light intensity on a short photoperiod 1 day after they had had enough light for induction. If the plants received 72 or more hours of continuous light the effect was apparently negligible. Evidently the light intensity was too low for promotion of the stimulus to flower after the first 24-hour period, and the sooner the plants were returned to short photoperiod where the light intensity was higher the more they responded by elongating. Also, continued exposure to the low light intensity may have depleted the carbohydrate reserve to the point where it was impossible for the plants to recover sufficiently to produce a vigorous flower stalk. If carbohydrate reserve was a limiting factor, it was just as limiting (expressed in terms of subsequent stem elongation) after 4 days of treatment as after 9. In addition it should be noted that, shortly after returning the plants to high intensity in the greenhouse, they recovered their green color rapidly and in most cases appeared normal.

SHORT PHOTOPERIODS OF LOW LIGHT INTENSITY FOLLOWED BY INTENSITIES VARYING FROM 50 TO 1000 FOOT-CANDLES.—Since dill showed practically no further response to continuous light of low intensity after being subjected to three or more 24-hour periods, it was thought possible to remove the effects of previous treatment with high light intensity by subjecting the plants to three short photoperiods of low light intensity (4–12 foot-candles). Accordingly on September 11, 1939, an experiment was started with plants thus treated, to determine the effect of various light intensities, supplied continuously for 4 days, on subsequent development.

The 175 plants were divided into seven equal groups, 25 in each group, and subjected to different intensities of light from Mazda filament lamps, which were placed at one end of a bench in the darkroom. Six groups of plants were arranged at intensities ranging from 50 to 1000 foot-candles. The seventh group was transferred to short photoperiod at the same time for use as a control. All the plants receiving continuous light at the different intensities for four 24-hour periods were returned to short photoperiod. This was done at 5:00 P.M. in order that the plants would receive a dark period immediately following the treatments. Some plants attained their maximum height and unfolded their inflorescences 51 days after the termination of treatment. Measurements were made at that time (fig. 4).

Up to 300 foot-candles, the average height attained was almost directly proportional to the intensity of light received; the average height of the plants receiving 500 and 1000 foot-candles was almost the same as that of the plants receiving 300. There is an apparent inhibitory effect or lack of stimulation of intensities higher than 300 foot-candles on the rate of elongation of the stems.

RED LIGHT AND NUMBER OF DAYS NECESSARY FOR INDUCTION.—Some work (21) has been done on the effect of red light on photoperiodic response when used to supplement natural day length. SCHAPPELLE (15), who worked with narrow ranges of wave lengths of radiant energy in the red portion of the spectrum, demonstrated with a number of species that when red light only is used during the photoinductive cycle, floral initiation will result. In order to determine whether red light of a number of different intensities would prove equally effective in inducing stem elongation in dill, an experiment was set up June 15, 1940.

The primary source of light was provided by Mazda filament lamps screened by using four thicknesses of red cellophane stretched over sheets of glass. These filters had a transmission range below 580 μ of 0 per cent and at 700 μ of 64 per cent. To eliminate most of the infra-red and minimize the amount of heat reaching the plants, 2-foot square, glass-bottomed trays about 4 inches deep were placed just below the reflectors and above the red filters. Two of these trays were filled with tap water, which flowed through them constantly. Two others, used for low intensities, had their water level maintained by frequent renewals. Four different intensities were used: 500, 250, 100, and 50 foot-candles. Under each of these, groups of twenty plants each were maintained on long photoperiods, each constituting a cycle of 20 hours of light and 4 hours of darkness.

Different groups of plants received one, two, three, four, and five long photoperiods at the various intensities. Treatment began at 5:00 P.M. after the plants had received 9 hours of daylight. At the conclusion of each of the treatments the plants received 20 hours of darkness and were then returned to short photoperiod. Stem development proceeded slowly until measurements were made on September 2 (fig. 5).

The results indicate that the several intensities provided during a long photoperiod are almost equally effective in influencing stem elongation. Intensities of 100, 250, and 500 foot-candles are relatively equally effective when given in two cycles, whereas two long photoperiods consisting of 50 foot-candles of red light given for 20 hours out of each 24 were more effective than a single cycle. All four intensities were about equally effective in initiating stem elongation when the induction treatment lasted 3 days. When the plants were subjected to four long photoperiods, the plants receiving 250 and 500 foot-candles of red light averaged 43–46 cm. high, while those receiving 50 and 100 foot-candles averaged 30–33 cm. high. There appeared to be a rather steady increase in average height of plants receiving 250 and 500 foot-candles of light with increasing number of photoinductive cycles. On the other hand, those plants receiving 50 and 100 foot-candles increased less in average height after they received three photoinductive cycles, the lack of increase being greatest where the intensity was lowest.

LOW TEMPERATURE DURING INDUCTION PERIOD AND FLOWERING RESPONSE.—The temperature at which *Xanthium* plants are maintained during the dark period is an important factor in determining the number of days required for induction to flower (9). Temperature during the photoperiod apparently alters the time required for induction very little. Since dill does not require a dark period for induction, a test was made to determine whether low temperature has any influence on the number of days required for induction.

One hundred and four plants growing on short photoperiod were selected June 15, 1939. These were divided into eight groups of thirteen each. One of these groups was maintained on short photoperiod as a control. The remaining groups were transferred to a room adjusted to 4.5° C. and controlled to within 0.5° C. Light of approximately 1800 foot-candles at the leaf surface was supplied by an Everready carbon arc lamp to give a photoperiod of 20 hours out of 24. After each photoperiod for the first 5 days, and then following the seventh and ninth photoperiods, one group was returned to short photoperiod in the greenhouse. They were maintained there until July 29, when measurements of the heights of stems were made (fig. 6).

The average height of the plants having had 2 days of cold treatment was greatest of any of the groups. Plants having had 3, 4, 5, and 7 days of the cold treatment were only approximately as tall as those receiving one, whereas those receiving nine cycles reacted as did the controls remaining on short photoperiod.

RESULTS FROM INJURY TO STEM.—Initial attempts to show that there was a transmittable flower-promoting substance in dill appeared promising. Further experiments were conducted with the introduction of a greater variety of controls. The data are briefly summarized as follows. One hundred and sixty-five plants were treated March 31 and April 1 in such a way that injury to the stem always resulted. Previous to the experiment, and for its duration, plants were maintained on a 9-hour photoperiod. Fifty-one plants were in flower at the conclusion of the experiment (May 25), the stems of ninety-eight had elongated, while only sixteen remained in the rosette condition. On the other hand, of the eighty-seven control plants one was in flower and the stems of seven had elongated.

The data indicate that injury to the stem results in a strong tendency to flower. Because of this, care must be exercised in transplanting and subsequent handling.

EXPERIMENTS WITH ANNUAL BEET

CONTINUOUS ILLUMINATION OF VARYING INTENSITIES FROM TIME OF GERMINATION.—Since dill, as well as a number of other long-day plants, will flower if kept on continuous day, beet was tested to see whether it required a dark period in order to bolt; that is, for the stem to elongate and to flower. Approximately 100 beet fruits were planted April 18, 1940, on the surface of the soil in flats and placed

in the greenhouse. Supplementary illumination was supplied by fluorescent Mazda lamps of the daylight type from 5:00 P.M. to 8:00 A.M., amounting to approximately 700 foot-candles at the surface of the soil. As the plants grew the reflector containing the fluorescent lamps was adjusted at higher levels. The intensity at the surface of the upper leaves, however, never fell below 200 foot-candles.

The seeds germinated rapidly and within 31 days the plants were producing flower stalks. As previously noted, dill which was growing under the same conditions required 10 days longer to produce inflorescences. Another point of contrast was that the leaves of beet grew large and succulent, whereas those of dill were small and expanded only slightly. Also the flower stalks of the beet were much taller than in dill, some of them attaining a height of over 100 cm., while those of dill averaged 10–15 cm.

Since variability of light intensity might be a factor involved in the speed with which beet responded to continuous illumination, an experiment was set up June 26, 1940, in a darkroom where the sole source of illumination was from fluorescent lamps. One set of lamps was adjusted to give 500 foot-candles at the surface of the soil, while the other gave slightly over 1000 at the same level. These lamps burned continuously and were not readjusted, so that as the plants grew in height the intensity at the leaf surface increased; the maximum attained was 1400 foot-candles. Temperature usually fluctuated about 4° C. during the day and ranged from 18° to 29° C. during the experiment.

When the beet fruits were planted, they were almost completely covered with soil and were thoroughly watered. Germination occurred a few days later. Twenty-eight days after the experiment was begun two of the plants receiving the highest intensity were developing inflorescences while the internodes of twenty-four had elongated. After 51 days the experiment under the highest intensity was terminated. By that time seventy-eight plants had bolted. The experiment under the lower light intensity was continued 25 days longer. At that time none of the plants had bolted. All grew vigorously vegetative, however, and had large deep green leaves.

CONTINUOUS ILLUMINATION AND NUMBER OF DAYS REQUIRED FOR INDUCTION.—It has been reported (**5**) that annual beet will flower if given fifteen or twenty photoperiodic cycles each consisting of 19 hours of light and 5 hours of darkness. To determine the effect of a longer photoperiod on the number of days for induction, 220 uniform plants were divided into two equal groups and transferred from cycles consisting of 8 hours of light and 16 hours of darkness to the following treatments. One group was placed on cycles of 21 hours of light and 3 hours of darkness; the second group was transferred to continuous light. After all the plants had received eight cycles of their respective treatments, ten were transferred each day from each of the two treatments to cycles consisting of 8 hours of light and 16

hours of dark. This was continued until the last group of plants had received eighteen cycles. Approximately a month later it was found that half the plants which had received continuous illumination for 11 days had bolted. Slightly more than half the plants receiving twelve cycles, each consisting of 21 hours of light and 3 hours of dark, had bolted. The internodes of such plants were relatively short and the leaves on the stems were larger than those on the plants which received thirteen or more cycles of either continuous light or 21 hours of light and 3 hours of dark. From their appearance the stalks seemed to be more leafy than completely induced plants. Bolting in plants having received 11 days of continuous light was little more pronounced as to height attained and was only a little more rapid than in plants which received the other treatment. The data indicate that thirteen or more cycles conducive to flowering are necessary before annual beet will be as completely induced, as shown by its bolting characteristics, as plants maintained continuously on cycles consisting of 21 hours of light and 3 hours of darkness.

INTENSITY OF LIGHT REQUIRED FOR INDUCTION.—In the open greenhouse, where beet was found to be induced on continuous photoperiod and on cycles consisting of 21 hours of light and 3 hours of darkness, the light intensities varied constantly during the day from 6000 foot-candles to approximately 100, and the intensity of supplementary illumination in the various experiments ranged from 70 to 750 foot-candles. There was also considerable diurnal fluctuation in temperature. Because of these variations in light and temperature, an experiment was designed to determine the minimum constant intensity of light which would induce flowering. Early in June forty-five uniform plants were selected from the short photoperiod bench, divided into three lots, and transferred to a basement room where they could be illuminated only by fluorescent lamps. Three arrangements were made, two provided 900 foot-candles at the leaf surface, the other 700. One of the former was regulated to provide cycles consisting of 20 hours of light and 4 hours of darkness, the other burned continuously. The one at 700 foot-candles also burned continuously. Temperatures ranged from 18° to 29° C. during the experiment.

Ninety-one days after the experiment began eleven of the plants receiving continuous light of 900 foot-candles had begun to flower or had elongated. Those exposed to cycles of 20 hours of light and 4 hours of dark responded more slowly, but nine of the fifteen did flower. Of the plants receiving 700 foot-candles continuously all but three remained vegetative. In another experiment, fifteen plants were maintained under conditions of continuous light at 700 foot-candles for more than 3 months, during which time but two plants flowered.

The minimum intensity of continuous light required for flowering in beet is approximately 700 foot-candles. If the intensity were to fall much lower the plants would probably all remain vegetative. Even at 700 foot-candles the plants re-

mained green for the duration of the experiment and appeared to be not greatly different from those grown in the greenhouse continuously. Some time after the plants were placed under the fluorescent lights, however, the newly forming leaves were a darker green than those grown in natural daylight and the plant had two or three fewer leaves, but the leaves were manufacturing sufficient food materials to store starch in the base of the expanded portion.

CRITICAL DAY LENGTH.—To determine the critical day length of annual beet, groups of fifteen plants were placed in small dark chambers (April 15, 1940) lighted by Mazda filament lamps providing 80–100 foot-candles at the leaf surface. The photoperiod consisted of 9 hours of natural daylight, supplemented as required to provide 9, 10, 11, 12, 13, 14, and 16-hour photoperiods out of each 24 hours. Two other groups of plants were used as controls. One of these was placed on long photoperiod of about 21 hours while the other received continuous illumination.

Within 20 days all the plants having continuous illumination and those receiving cycles of 21 hours of light and 3 hours of darkness were flowering. Twenty-four days later (June 12) four plants receiving cycles of 16 hours of light and 8 hours of darkness were bolting. By July 21 the remaining eleven plants had flowered or were showing some elongation. On this date four of the fifteen plants receiving cycles of 14 hours of light and 10 hours of darkness were flowering or were elongating. There was no flowering in any of the other treatments. The data indicate that the critical day length for this variety of beet lies between 13 and 14 hours; that is, it does not flower if subjected to more than 9 or 10 hours of darkness in each 24-hour period.

Light intensity is probably an important factor in determining critical day length and in influencing the time necessary for response. On July 9, when the day length was 14 hours and 54 minutes, 120 plants were divided equally into four groups. One group received 21 hours and 37 minutes of light per day, a second 20 hours and 37 minutes, a third 19 hours and 37 minutes, and a fourth natural day length. Illumination supplemental to natural day length was provided by Mazda filament lamps. Owing to the decreasing duration of light per day, the total amount of light received at the end of the experiment was considerably less than at the beginning. Only 17 days later the plants in all three groups receiving supplementary illumination were bolting. Eleven days later nineteen out of thirty of the plants receiving natural day length were bolting.

The indication from a comparison of the speed with which beet responded to natural day length and to 9 hours of sunlight plus 7 hours of Mazda filament light is that either intensity or quality of light given after 9 hours of daylight is important in governing the rapidity with which bolting is induced.

"CUMULATIVE EFFECTS" OF EXPOSURE TO LIGHT.—There has been abundant evidence on the cumulative effect of photoperiodically inductive cycles on subsequent response to both long and short-day plants. This point is well illustrated by those plants which require more than one photoperiod of a definite length for induction. In some plants the requisite number of photoperiodic cycles for induction must be given without interruption. Biloxi soybean is one of these. Under ordinary circumstances it flowers only after exposure to four consecutive cycles consisting of 13 hours of light and 11 hours of dark. LONG (**9**) demonstrated the presence of cumulative effects, which endured for 24 hours, in *Xanthium*. Evidence from experiments with beet, however, indicates that the effects of ten consecutive cycles consisting of 18–20 hours of light and 6–4 hours of dark may last as long as 16 days, at which time four or five more long photoperiods consisting of 18–20 hours of light out of 24 result in flowering (**5**).

In order further to examine the tendency of beet to show this so-called cumulative effect of long photoperiods, a number of experiments were performed. Some of these involved the use of cycles of light and dark of equal duration, giving a total of 12 hours of light and 12 hours of dark. GARNER and ALLARD (**3**) found that if long-day plants were given cycles of light and dark ranging from 6 hours' light and 6 hours' dark to 1 minute light and 1 minute dark, they would respond as though on long photoperiod even though the total amount of light received was less than required for flowering if given continuously.

The outline of procedure was as follows: (*a*) 5 minutes of light and 5 minutes of dark; (*b*) 30 minutes of light and 30 minutes of dark; (*c*) 24 hours of light and 24 hours of dark. Other experiments involved cycles in which the light and dark periods were not of equal duration. One of these was designed to give one cycle with 8 hours of light and 16 hours of dark, followed by one cycle with 21 hours of light and 3 hours of dark; and then each cycle was repeated in order. Another similar experiment was performed in which three cycles, each consisting of 8 hours of light and 16 hours of dark, were followed by three cycles each consisting of 21 hours of light and 3 hours of dark. Flowering occurred in none of these experiments, even though the minimum duration of such continuous treatment in any instance was 2 months.

It has been reported that at least one long-day plant, dill, can be induced to flower by exposing a single leaf to conditions of long photoperiod (**7**). Repeated attempts have been made to cause beet to bolt by exposing a single leaf to conditions of long photoperiod, but in no instance has this resulted.

A number of experiments with short-day plants have shown that the physiological conditions involved in induction can be transmitted from one plant to another of the same species (**6, 11, 12**). In some of MELCHERS' work (**12**) involving the grafting of different species, a vegetative plant was induced to flower when it was

grafted to a different species which was flowering. If other vegetative plants could be thus induced to flower, considerable affirmative evidence could be brought to bear on the hormone theory of floral initiation.

Numerous grafts between different varieties and species were made. Among the grafts tried was one in which annual beet, of an unnamed variety closely related to biennial beet, was approach grafted, in the region of the hypocotyl, to biennial beet. Immediately after grafting the two varieties, they were placed on the long-day bench. After 3 weeks the annual beets were flowering vigorously. Although the grafts took firmly, the biennial beets remained vegetative throughout a period of 2 months, when the experiment was discontinued. All evidence from the grafting experiments indicated that if there was a flower-inducing substance transferred from one plant to another, the biennial variety still did not flower.

Discussion

With an increase of critical data on floral initiation and vernalization processes, sufficient information is now available to permit some speculation as to the phases involved. Three of the more direct hypotheses which have arisen as a result of vernalization and photoperiodic work have been those of GREGORY and PURVIS (4), MELCHERS (12), and HAMNER (5).

It is significant that while these workers have advanced their theories independently and have used different lines of approach, their conclusions are fundamentally similar. They all agree that there is some substance or physiological condition necessary before photoperiod becomes an active agent in altering the plant's physiology sufficiently to induce it to change from the vegetative to the reproductive state. They also agree that photoperiodic cycles of light and dark can then determine the production of another substance or alter the physiological condition sufficiently to bring about the initiation of floral primordia.

HAMNER (5) has conducted a number of experiments bearing on the subject of floral initiation in two short-day plants, cocklebur and Biloxi soybean. While he is conservative in his interpretations of the substances or conditions involved, he demonstrated that conditions previous to photoperiodic induction could limit, to a certain extent, various activities which occurred during induction. Of course, this might be interpreted in terms of MELCHERS' hypothesis to indicate that the degree or amount of vernalin present had been affected previous to the photoperiodic inductive treatment.

HAMNER was chiefly concerned in his interpretation of the causes related to floral initiation and with the importance of not only the light and dark phases of photoperiodic cycles leading to induction but also of the conditions which prevailed subsequent to the imposition of such cycles or series of cycles. From his work and that of his coworkers, he suggested that there was a physiological condi-

tion or change brought about during the light period which he designated as A; others which took place in darkness were designated as B; and those which might be considered as post inductive were termed C. For brevity of reference he used the progression A, B→C to indicate the entire interrelationship.

In previous experiments involving reactions to 14 hours of light followed by 10 hours of darkness, dill remained in the rosette condition. In these experiments it was found that if the cycle was 9 hours of light and 7 hours of dark, then 1 hour of light and 7 hours of dark, the plants responded by elongating their stems. If more than 1 hour of light was given in the middle of the dark period the stems elongated at a rate almost proportional to the duration of the interposed light phase. This suggests that there is a tendency for dill to assume a physiological condition which becomes increasingly strong for the promotion of stem elongation during the photoperiod, but that during a dark period longer than the critical there is a retrogression to the condition prevailing previous to illumination. This retrogression may be slow and require the full dark period. Light may arrest it. If the dark period is interrupted, therefore, retrogression in this physiological tendency is greatly lessened and the degree to which it is halted is roughly proportional to the duration of light received during the dark period.

That there is a tendency for accumulation of small amounts of some kind of flower-forming stimulus in dill, even though it is on cycles consisting of 9 hours of light and 15 hours of dark, is indicated by the fact that after approximately 9 months of such cycles all of a group of fifty plants flowered within approximately 1 week. The tendency for darkness, in excess of the critical, to retard development of dill as well as other long-day plants may be of varying effectiveness. MURNEEK (13) points out that *Rudbeckia* may be maintained in a rosette condition on photoperiods of 12 hours or less for more than a year, provided the temperature does not become extreme. High temperature, however, will overcome the retarding effect of darkness, and this plant will then react—so far as reproductive response is concerned—as an indeterminate.

In an experiment designed to determine the critical day length of annual beet, further evidence was given for the accumulation of a flower-promoting stimulus. On continuous day beet began to show some stem elongation about 14–16 days after treatment began. Those plants receiving a 21-hour photoperiod began to bolt a few days later. It was not until 12 weeks after the experiment had begun that all the plants having cycles, each consisting of 16 hours of light and 8 hours of darkness, had bolted. After the same interval of time four of the fifteen plants receiving cycles of 14 hours of light and 10 hours of dark had bolted. While this experiment is not conclusive, it appears that over a period of time there is a slow accumulation of the residual effects of light which is slightly in excess of the

critical day length, ultimately reaching a threshold value which results in stem elongation and floral initiation.

Intensity of light was shown to play an important role in altering the physiological state of both dill and beet sufficiently to bring about flowering. In an experiment with dill it was found that when different groups of plants were exposed to different intensities of continuous light, their response in stem elongation was almost proportional, up to 300 foot-candles, to the amount of light received. With beet the plants flowered only after receiving light of 900 foot-candles for 6 weeks. Plants similarly treated, except that the light intensity was 700 foot-candles, flowered in only two cases out of fifteen.

Another environmental factor of importance in altering the response of dill to long photoperiod was temperature. If plants having cycles each consisting of 20 hours of light and 4 hours of darkness were maintained at a temperature of 4.5° C. they were only slightly responsive. The longer they received this treatment the less response they gave. From a theoretical viewpoint this would seem to indicate that higher temperatures are necessary for the efficient change of physiological conditions sufficiently to bring about stem elongation and flowering.

In general it appears that in dill—even under conditions of long photoperiod or in some instances continuous light—the greater the duration (at least beyond 1, 2, and 3 days) of such conditions as exposure to 4 foot-candles of Mazda filament light, temperatures of 4.5° C., and intensities of red light of 50 foot-candles, the weaker the response in terms of average elongation of stems.

Summary

1. If maintained on continuous light in the greenhouse, dill flowered in 40 days after sowing the seeds and beet in 31 days. Under conditions of relatively constant illumination at 1000–1400 foot-candles, dill required 40 days from time of planting to show some stem elongation; approximately 20 more days were required to bring about the same response under 500 foot-candles. Under similar conditions of constant illumination of high intensity, beet produced seed stalks 28 days after planting. On the other hand, beet grown under 500 foot-candles did not produce seed stalks.

2. With increasing amounts of illumination during the middle of the dark period, up to 6 hours, the average height of the dill plants was almost directly proportional to the amount of supplementary illumination received.

3. Under conditions of high light intensity, dill may be induced to flower by giving it one long photoperiod consisting of 20–21 hours of light and 4–3 hours of dark. Rapidity of stem elongation was about the same when the plants received one, two, or three long photoperiods. But if the plants received four long photoperiods, they elongated about as rapidly as those maintained on long day.

4. Dill plants previously treated with a high light intensity showed a response to continuous illumination of only 5 foot-candles if exposed to it for as little as 1 day. Those subjected to this treatment for 2 days showed some response, while those having 3–8 days of low light continuously were very slow in responding.

5. When dill was pre-treated with three short photoperiods each consisting of 9 hours of light of 4–12 foot-candles and 15 hours of darkness, and then subjected to intensities of 50–1000 foot-candles, there was a steady increase in average height with increase in intensity up to 300 foot-candles. The effect of higher intensities (500–1000 foot-candles) on rate of stem elongation was approximately the same as that produced by 200 foot-candles.

6. The effect of low temperature (4.5° C.) on dill plants receiving cycles consisting of 20 hours of light and 4 hours of darkness was to suppress markedly the flowering response. Those plants receiving 2 days of cold treatment attained a greater height than did any of those receiving fewer or more days of such treatment.

7. A large proportion of young dill plants elongated their stems if the roots or stems were injured. Thus injury seemingly altered the metabolism sufficiently to bring about the flowering response.

8. Red light of wave lengths greater than 580 μ are capable of inducing the flowering response in dill when used as the sole source of illumination during the induction period.

9. Although red light of 50 foot-candles was approximately as effective in bringing about induction in dill as any intensity up to 500 foot-candles, it was not nearly so effective after 5 days of treatment as the higher intensities.

10. Annual beet did not flower if it received more than 10–11 hours of darkness in each 24.

11. Annual beet began to bolt after return to short day if it received 11 days of continuous light. The bolting of such plants, however, was no more rapid or pronounced than in those receiving cycles consisting of 21 hours of light and 3 hours of darkness.

12. Continuous illumination with 900–1000 foot-candles of light from white fluorescent Mazda lamps, under conditions of only slight temperature fluctuation, was conducive to flowering in beet. If the intensity did not rise above 700 foot-candles during the induction period, flowering did not occur.

13. Annual beet did not flower if subjected to the following cycles of light and dark: (*a*) 5 minutes of light and 5 minutes of dark (55 days); (*b*) 30 minutes of light and 30 minutes of dark (55 days); (*c*) 24 hours of light and 24 hours of dark (117 days); (*d*) one short photoperiod of 8 hours of light, 16 hours dark and one long photoperiod of 21 hours of light, 3 hours dark (117 days); (*e*) three short

photoperiods of 8 hours of light, 16 hours dark and three long photoperiods of 21 hours of light, 3 hours dark (117 days).

14. Exposure of a single leaf of beet to cycles of 20 hours of light and 4 hours of darkness did not result in the plant flowering.

The writer wishes to express his appreciation of the helpful suggestions and continued interest of Dr. K. C. HAMNER during the course of these experiments.

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EFFECTS OF ROOT-GROWTH HORMONES ON THE MERISTEM OF EXCISED PEA ROOTS[1]

FREDRICK T. ADDICOTT

(WITH SIX FIGURES)

Introduction

Roots of pea, radish, flax, and tomato, whose nutrition has been studied by experiments with cultures in vitro, require vitamin B_1 as an accessory growth factor (**4, 7, 6**). For maximum growth certain of these roots (pea, tomato, radish) require in addition nicotinic acid (**2, 6**). Since in the plant vitamin B_1 and nicotinic acid have all the characteristics of hormones, it seemed of interest to investigate their histological effects. This paper presents observations of the meristems of excised pea roots cultured under conditions of deficiency of vitamin B_1 or of nicotinic acid. The anatomical effects of vitamin B_1 deficiency on isolated pea roots has been described in an earlier paper (**1**). It was found that in the absence of vitamin B_1 from the culture medium, cell divisions in the apical meristem of the root cease. The roots used in the earlier investigations, however, were cultured in medium containing no nicotinic acid. Such roots suffer primarily from deficiency of vitamin B_1, but the symptoms observed might have been attributable in part to deficiency of nicotinic acid. It seemed advisable, therefore, to examine roots in which these two deficiencies were present independently.

Roots of *Pisum sativum*, variety Perfection, were cultured by methods previously described (**5, 3**) for five weekly transfers. The experiment proper was not started until after the fifth transfer, in order to insure that the roots were well established. After this time roots receiving both vitamin B_1 and nicotinic acid in addition to salts maintain a uniform appearance and growth rate in all succeeding transfers. Two hundred and fifty roots, which had averaged 75.6 mm. of growth during the fifth week, were selected. These were divided into three lots: one received vitamin B_1 as the only accessory growth substance; a second received only nicotinic acid; and the third, the control group, received both substances. Growth was measured at the time of each weekly transfer, and as the effects of the deficiencies became apparent, samples of the roots were fixed for sectioning and further study. The material was fixed in Navashin's solution and always at noon, in order to eliminate variation in the number of cell divisions which might result

[1] Work carried out with assistance from the Works Progress Administration, Official Project 665-07-3-83, W.P. L-9809.

from fixation at different stages in the cycle of diurnal fluctuation of mitosis. The root tips were dehydrated with tertiary butyl alcohol and sectioned in paraffin. Sections were stained with iron-haematoxylin.

Observations

The growth rate of roots receiving only vitamin B_1 or only nicotinic acid declines and in the course of a few weeks reaches zero. In the absence of vitamin B_1 growth falls off more rapidly than in the absence of nicotinic acid (table 1). This accounts for the fact that vitamin B_1 was the first accessory growth factor discovered for excised pea roots. Figures 1–3 show the habit of the roots in culture and that deficiency of vitamin B_1 reduces growth rate to a greater extent than does deficiency of nicotinic acid. Roots lacking nicotinic acid become thin, particularly toward the apex; those lacking vitamin B_1 remain thick and frequently show irregular swellings.

TABLE 1

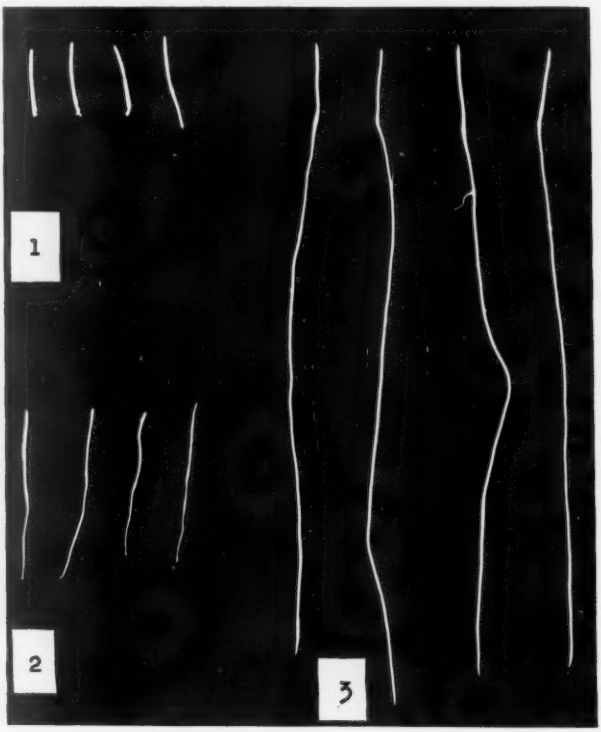
GROWTH OF PEA ROOTS IN ABSENCE OF VITAMIN B_1 OR NICOTINIC ACID

ACCESSORY SUBSTANCES IN MEDIUM	GROWTH RATE IN MM. PER WEEK				
	5TH*	6TH	7TH	8TH	9TH
Vitamin B_1 plus nicotinic acid	75.6	81.0	80.3	78.2	82.4
Vitamin B_1 alone		77.4	55.9	32.2	17.8
Nicotinic acid alone		79.1	59.8	22.2	8.2

* Roots cultured in medium containing vitamin B_1 and nicotinic acid during the first 5 weeks.

A preliminary examination of the sections showed that roots deficient in vitamin B_1 and those deficient in nicotinic acid differed not only from the controls but also from each other. The length of the meristem and the number of dividing cells it contained, as well as the width of the meristem and the number of columns of cells, were all affected by both deficiencies. Both deficiencies also affected cell elongation to some degree. The formation of starch grains and the deposition of cell-wall materials appeared to be unaffected, at least during the early stages. In order to obtain a quantitative basis for the comparison of the roots in this experiment, measurements were made of the affected anatomical features just mentioned. For this purpose median longitudinal sections were used, and counts were made from the two sections nearest the midline of each root. The results are summarized in table 2. The measurements show that in the case of both deficiencies the decline in growth rate is accompanied by a decrease in the length of the

meristem as well as in the number of its cell divisions. In 4 weeks the growth rate of roots deficient in nicotinic acid drops to about 20 per cent of that of the controls (table 1), while the meristem becomes shorter in nearly the same degree and the number of cell divisions in the meristem falls to 40 per cent of the number of the control. In the same period vitamin B_1-deficient roots drop in growth rate to



FIGS. 1–3.—Shadow photographs of roots at end of ninth weekly transfer: Fig. 1, from medium lacking vitamin B_1; fig. 2, from medium lacking nicotinic acid; fig. 3, control from medium containing both growth substances.

10 per cent of the rate of the controls, while the meristem becomes reduced to about 15 per cent of the length of the controls and the number of cell divisions also decreases in about the same proportion.

It is also evident from examination and measurement of the more proximal regions of the sections that the cells of both types of deficient roots do not elongate to so great an extent as do those of the control roots which received both growth substances. Both types show elongation to only about 60 per cent of the length

of the controls. The measurements were made from cells 5–6 mm. from the apex of the roots. These portions of the different roots are not strictly comparable. The deficient roots were growing at a much slower rate, thus cells 5 mm. from the apex had had more time in which to elongate than had cells the same distance from the apex of the rapidly growing roots. It appears probable, therefore, that if fully mature regions were compared, the cells of deficient roots would be found to be considerably less than 60 per cent of the length of cells in roots receiving both vitamin B_1 and nicotinic acid.

TABLE 2

MEASUREMENTS TAKEN FROM SECTIONS OF PEA ROOTS DEFICIENT IN VITAMIN B_1 OR NICOTINIC ACID

Accessory growth substances in medium	Week of fixation	Width of meristem*			Length of meristem† (mm.)	No. of cell divisions in meristem‡	Length of cortical cells§ (mm.)
		in mm.	in terms of cells	Average cell width (mm.)			
Vitamin B_1 and nicotinic acid (controls)	8th and 9th	0.392	37.2	0.0106	1.50	11.9	14.3
Vitamin B_1 alone	8th	0.310	36.3	0.0085	0.87	11.9	10.3
	9th	0.260	32.3	0.0081	0.40	4.6	8.0
Nicotinic acid alone	8th	0.384	37.9	0.0101	0.81	6.9	9.3
	9th	0.359	37.0	0.0097	0.20	1.7	8.9

* Measurements of width taken at proximal end of meristem, at point where cell divisions cease.

† From tip of meristem to last observable mitotic figure that was part of apical meristem.

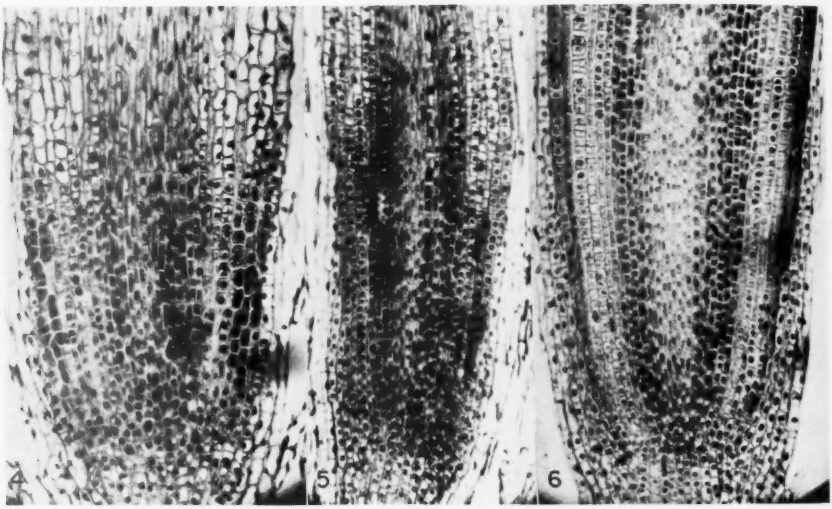
‡ These figures include only the number of cells in metaphase or anaphase to be found in median longitudinal sections. They are a measure of the number of cell divisions in the root.

§ Longest cortical cells in the region 5–6 mm. from tip measured.

The meristems of the deficient roots differ from each other also in diameter (figs. 1–6). Nicotinic acid-deficient roots averaged 65 per cent of the diameter of the controls, while vitamin B_1-deficient roots averaged slightly over 90 per cent of the controls. The diameter was also measured in terms of cells. All measurements were made at the proximal portion of the meristem, where cell divisions cease. The thinness of the nicotinic acid-deficient roots was correlated with the smaller size of their cells and the fact that such roots contain fewer columns of cells than do the control roots. The cells of the nicotinic acid-deficient roots averaged about 75 per cent of the diameter of similar cells of the control roots, and the sections of the deficient roots contained only 85 per cent as many columns of cells as did the controls. The thickness of the meristem of vitamin B_1-deficient roots is only slightly less than that of the controls. The number of columns of cells in these meristems is the same as that of the controls, but the average diameter of the cells, being about 90 per cent of the controls, accounts for the slightly thinner

overall diameter of the vitamin B_1-deficient roots. As noted, the nicotinic acid-deficient roots remain smooth while vitamin B_1-deficient roots often develop irregular swellings. No detailed examination was made of these swellings, but from limited observations it appeared that they are due to a radial enlargement of groups of cortical cells.

Roots in the most advanced stages of deficiency studied, that is, those cultivated in deficient medium for 4 weeks, showed considerable starch deposition as well as every indication of active cell-wall formation. Starch grains are conspicu-



FIGS. 4–6.—Sections of roots made at end of ninth weekly transfer: Fig. 4, cultured in medium lacking vitamin B_1; fig. 5, in medium lacking nicotinic acid; fig. 6, control cultured in medium containing both growth substances.

ous in the region of the meristem, especially in the vacuolated cells of the epidermis and root cap. They are rarely seen in similar regions of the actively growing control roots. Likewise the cell walls of both types of deficient roots appear to be well formed and heavy, especially in the root cap and peripheral regions of the meristem. In the deficient roots also there is a strong tendency for the root cap to adhere to the tip. It is doubtful whether root-cap formation is more extensive in the deficient roots, since the controls regularly form large masses of cap tissue. In the latter case, however, the caps rarely remain attached to the roots after the process of fixation and dehydration. Figures 4–6 show the topography of the affected roots, their relative diameters, and the extent of the meristematic regions after 4 weeks in deficient medium. Portions of the root cap can be seen adhering to the roots cultured in deficient media.

Summary

1. Deficiencies of the root-growth hormones vitamin B_1 and nicotinic acid in cultures of excised pea roots have visible effects in the meristem.

2. The deficiency of either substance results in a decrease in the growth rate and eventual complete cessation of growth. This is accompanied by reduction in the length of the meristem, decrease in the number of cell divisions in the meristem, as well as reduction of the total length attained by the cells as they mature.

3. Roots deficient in nicotinic acid become thin. This is accompanied by reduction of the diameter of the root cells and of the number of columns of cells in the roots.

4. The meristems of roots deficient in vitamin B_1 become only slightly smaller in diameter in the course of 4 weeks, but their growth rate declines more rapidly than does that of roots deficient in nicotinic acid. The mature portions of these roots develop irregular thickenings.

Most of this work was done at the California Institute of Technology, and the writer is indebted to Dr. JAMES BONNER and Dr. F. W. WENT for helpful suggestions during the course of the investigation.

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GROWTH OF SOME RANGE GRASSES IN REDUCED LIGHT INTENSITIES AT CHEYENNE, WYOMING

H. M. BENEDICT

(WITH FOUR FIGURES)

Introduction

Several investigations (**10, 4, 1, 3, 9**) have been carried out on the growth of plants in full sunlight and shade, but only two of them—one dealing with sunflowers (**5**) and the other with trees (**8**)—have been conducted in regions where the intensity of full sunlight is greater than 10,000 foot-candles. At Cheyenne, Wyoming, sunlight intensities as high as 14,000 foot-candles (as measured by a Weston meter) have been recorded, and readings of 12,000 are common on clear days. Repeated readings taken at random between 9:00 A.M. and 3:00 P.M. from June to September, inclusive, have almost always shown the intensity to be above 10,000 foot-candles on cloudless days. Since such intensities are higher than reported in some other sections of the country, the question was raised as to whether or not these intensities were too high for the best growth of some of the range grasses.

This paper presents the results obtained when plants of crested wheat grass (*Agropyron cristatum* (L.) Gaertn.), bluestem (*A. smithii* Rydb.), and blue grama (*Bouteloua gracilis* (H.B.K.) Lag.) were grown in full sunlight and under shades transmitting various percentages of full sunlight but not changing the day length.

Methods

Seed of the various species, gathered the previous year from plants growing in the grass nursery at Cheyenne, were planted in boxes $1 \times 1 \times 1$ feet, filled with soil. After the seed had germinated the seedlings were thinned to twenty-four per box and grown until the end of the experiment. The soil was then carefully washed from the plants and their dry weight determined by drying to a constant weight at 100° C. In 1938 the seed was planted June 7 and the plants harvested October 1. In 1939 the seed was planted June 14 and the plants harvested October 3.

Four intensities were used in 1938: 100 per cent, 57 per cent, 42 per cent, and 28 per cent of full sunlight. The shaded plants were grown in a lathhouse under none, one, and two layers of ordinary wire window screen. The shaded plants were about 20 feet from the unshaded ones, but this difference in location probably had no appreciable effect on their growth.

Because of the wide gap between 100 per cent and 57 per cent sunlight, and be-

cause it was possible that the wide bands of full light and shade passing across the leaves of the plants growing in the lathhouse might have had some effect on the results, the experiment was repeated in the summer of 1939, with the following modifications. In the 1939 series the plants were grown in the open in full sunlight and in the shade cast by one and two layers of window screen or in 100 per cent, 70 per cent, and 50 per cent of sunlight intensity, respectively. In this series the different treatments were randomized, thus eliminating the possibility of any positional effect being responsible for the differences occurring in the treatments.

In order to shade the plants with the window screen, wooden frames 4×4×4 feet were constructed and one or two layers of screening secured to four of the six faces. These screened frames were then placed over groups of nine boxes, three boxes of each species, one open side on the ground and one facing north. Thus the plants were shaded from above and on the east, south, and west sides, leaving the north side open, so that access could be had to the plants. Except for short periods in early morning and late evening during the early part of the summer, this kept them shaded at all times from the sun.

The values given for the percentages of full sunlight transmitted by the various screens are those obtained when the screens were placed at right angles to the direction of the sun's rays. GARNER and ALLARD (7) have pointed out that the percentage transmission of screens of the type used in these experiments varies with the angle at which the light strikes the screen, the greatest transmission occurring when the light is traveling at right angles to the screen. The relative light intensities in the various treatments were checked once a week with the Weston meter.

In both years the soil used was carefully mixed to insure uniformity among treatments. In 1938 the boxes were all filled to the same level, so that approximately the same amount of soil was placed in each flat. In 1939 the same weighed amount was placed in each flat. The flats were watered whenever they required it. Of course the plants in the reduced light intensity required watering less often than those in full sunlight. The flats of the 1939 series were weighed occasionally during the summer, and at no time was there any difference in the weights of the flats in the various intensities. Since the same amount of soil was placed in each box at the beginning of the experiment, and assuming the weights of the plants to be negligible, evidently the amount of moisture available to the plants was approximately the same in all treatments. While these weights were not taken in 1938, the same method of watering was employed, and presumably there was no appreciable difference during that year. In neither year did the plants in any treatment wilt.

It was never possible to demonstrate a difference in air temperature in any of the treatments in either year. Soil temperatures taken 2 inches beneath the surface were 1–2° C. higher in the flats exposed to full sunlight than in those that were

shaded. While these differences in soil temperature were consistent, they were not great enough to account for the differences obtained between the plants in the different treatments.

Results

The results are the means of nine replications for 1938 data and six for 1939 data, each replication consisting of twenty-four plants. Thus in 1938 there were 216 and in 1939, 144 plants of each species in each treatment.

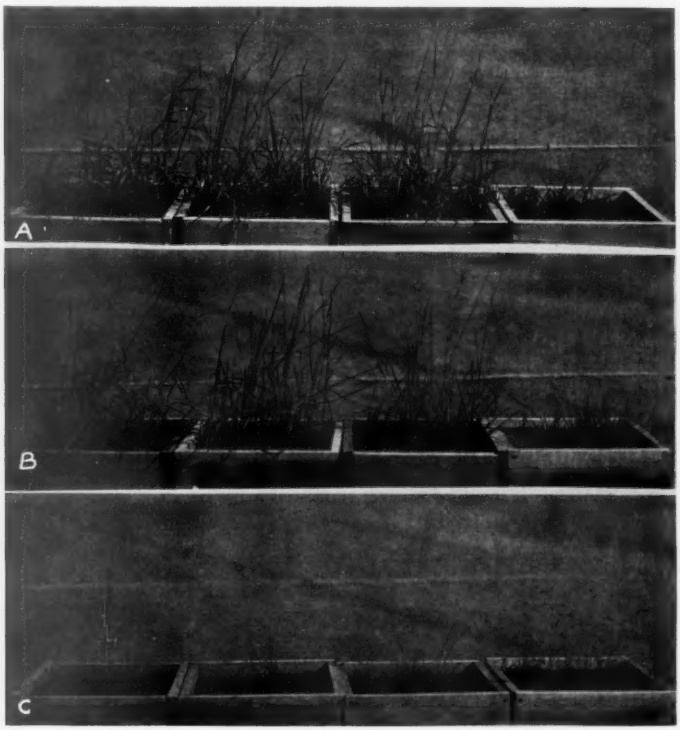
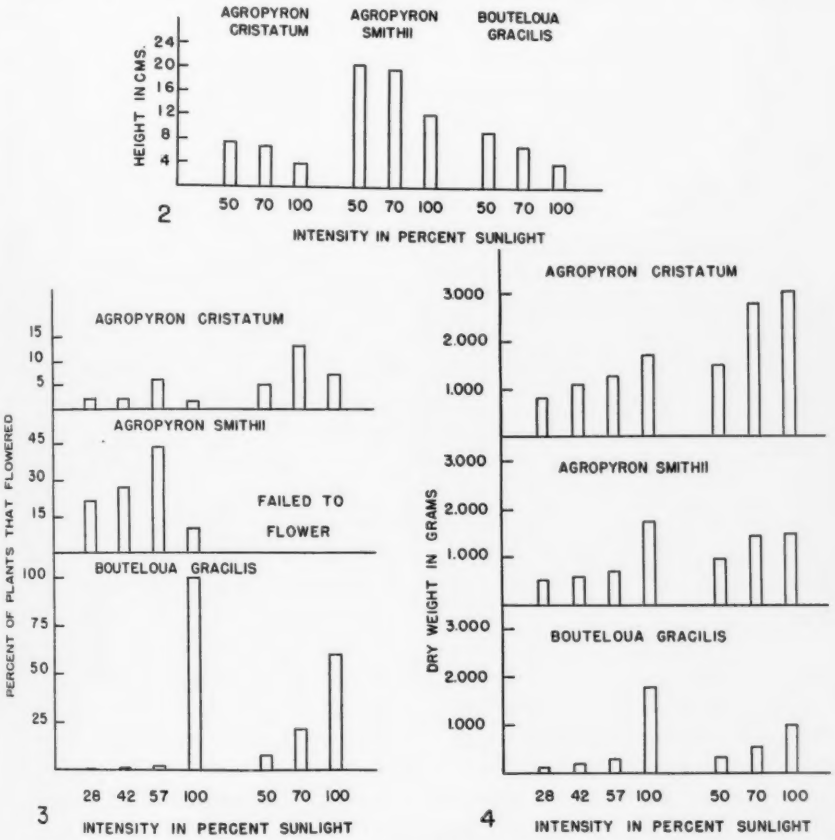


FIG. 1.—Plants grown in (left to right) 28 per cent, 42 per cent, 57 per cent, and 100 per cent sunlight in 1938: *A*, *Agropyron cristatum; B*, *A. smithii; C*, *Bouteloua gracilis*.

Representative plants in the different treatments at the end of the summer of 1938 are shown in figure 1. The shaded plants elongated and became spindly, while the unshaded plants were short and assumed an almost rosette type of vegetative growth. Photographs of the plants grown in 1939 are not shown, but the same type of growth was noted in both the shaded and unshaded plants.

The heights of the plants were not recorded in 1938, but figure 1 indicates that

as the light intensity was reduced the height of the vegetative parts of the plants of the three species increased to a maximum and then decreased, the greatest height being reached at 42 per cent full sunlight. Plants grown in 70 per cent and



FIGS. 2–4.—Fig. 2, height of plants grown in different light intensities in 1939. Fig. 3, number of plants that bloomed in different light intensities in 1938 (left) and 1939 (right). Fig. 4, dry weights of entire plants grown in different light intensities in 1938 (left) and 1939 (right).

50 per cent sunlight were always taller than those grown in 100 per cent (fig. 2), but the tallest plants of all species were in 50 per cent sunlight. These figures are in general agreement with the observations made on the heights of the plants grown in 1938.

Counts were made of the number of plants that flowered in the various treatments, and the results, calculated in percentage of the total number of plants in

each treatment, are shown in figure 3. A greater percentage of plants of *Bouteloua gracilis* and a smaller percentage of *Agropyron cristatum* and *A. smithii* flowered in 100 per cent sunlight than flowered in the reduced light intensities. Also the seed heads of *A. cristatum* and *A. smithii* were much larger on the plants growing in the shade than on those in full sunlight, regardless of the percentage of plants that flowered. Plants of *B. gracilis*, however, seemed to have the largest seed heads when grown in 100 per cent sunlight. No measurements were made on the actual

TABLE 1

DRY WEIGHTS IN GRAMS AND ROOT-TOP RATIOS OF PLANTS GROWN IN DIFFERENT LIGHT INTENSITIES

PART OF PLANT AND SPECIES	LIGHT INTENSITY AS PERCENTAGE OF FULL SUNLIGHT						
	1938				1939		
	28	42	57	100	50	70	100
Roots:							
Agropyron cristatum	0.410	0.627	0.801	0.945	0.783	1.312	1.477
Agropyron smithii	0.220	0.240	0.310	1.075	0.520	0.902	0.989
Bouteloua gracilis	0.046	0.096	0.137	0.733	0.133	0.231	0.360
Tops:							
Agropyron cristatum	0.397	0.460	0.450	0.801	0.701	1.457	1.529
Agropyron smithii	0.317	0.325	0.397	0.651	0.434	0.529	0.482
Bouteloua gracilis	0.052	0.097	0.157	1.050	0.200	0.327	0.641
Total plant:							
Agropyron cristatum	0.807	1.087	1.251	1.746	1.484	2.769	3.006
Agropyron smithii	0.537	0.565	0.707	1.726	0.954	1.431	1.471
Bouteloua gracilis	0.098	0.193	0.294	1.783	0.333	0.558	1.001
Root-top ratio:							
Agropyron cristatum	1.038	1.384	1.773	1.309	1.080	0.940	0.973
Agropyron smithii	0.657	0.741	0.811	1.761	1.273	1.753	2.162
Bouteloua gracilis	0.921	1.094	0.960	0.803	0.687	0.732	0.560

size of the heads, but some idea of the differences may be seen from the plants of *A. cristatum* and *B. gracilis* in figure 1.

In 1939 the seed of *A. smithii* was extremely slow in germinating, with the result that the plants had made almost no growth by July 15. Unpublished data (2) have shown this to be a long-day plant (**6**), and it may be that this species failed to flower because by the time the plants were large enough to be responsive to the photoperiodic stimulus, the days long enough to induce flowering had passed.

The dry weights of the plants in the various treatments are shown in table 1 and in figure 4. In general these results indicate that these species make their best growth in greater than 70 per cent of the full sunlight at Cheyenne, and that as the light intensity is decreased, at least below 70 per cent sunlight, the dry weights of

the plants decrease. They also show that reductions in light intensity are accompanied by a greater decrease in growth of *B. gracilis* than of *A. cristatum* and *A. smithii*.

The root-top ratios obtained from the dry weights as shown in table 1 indicate that reductions in the intensity of full sunlight by certain amounts may cause a smaller decrease in the growth of the roots than of the tops of *A. cristatum* and *B. gracilis*. They also show that any of the intensities used in these experiments cause a greater decrease in the growth of the roots than of the tops of *A. smithii*.

The ratios presented are not those that would be obtained by dividing the average weight of the roots by the average weight of the tops, but are the means of nine or six ratios (depending on the year) yielded by the individual replications.

Discussion

The increase in height and the decrease in dry weight of the plants grown in the shade as compared with those grown in full sunlight are in general agreement with the results obtained by others, as reviewed by SHIRLEY (10) and BURKHOLDER (4). However, the dry weights of the plants of *Agropyron cristatum* and *A. smithii* in 100 per cent sunlight were not much greater (less in the case of the tops of *A. smithii*) than in 70 per cent sunlight. This indicates the possibility that in 80 per cent or 90 per cent sunlight the dry weights of at least two species might be equal to if not greater than those in 100 per cent sunlight.

The marked results of the shading in increasing the number of plants of *A. cristatum* and *A. smithii* that flowered indicate that, in the vicinity where these experiments were carried out, 100 per cent sunlight is so intense as to be unfavorable for the flowering of these species, at least in the first year of their growth. This may partly explain why plants of these two species flower so sparsely in field and nursery plantings during their first growing season. On the other hand, the great decrease in the number of flowering plants of *B. gracilis* in the reduced light intensities seems to show that 100 per cent sunlight is the most favorable intensity for the blooming of this species. Field and nursery plantings of this species usually flower abundantly during their first year.

The difference in blooming response and the small reduction in dry weight in the shade of plants of *A. cristatum* and *A. smithii* as compared to plants of *B. gracilis* cannot be explained on the basis of the native habitats of the species, since all three normally occur and are grown in dry plains exposed to the full intensity of the sun.

The similarity in response of the plants in the reduced light intensities in 1938 and 1939 seems to indicate that there is little if any difference between the type of shade cast by wide strips of opaque material and the type cast by window screen, as far as its effect on plant growth is concerned.

In some work on *A. smithii* and *B. gracilis* to be published elsewhere (**2**), and in some unpublished data on *A. cristatum*, it is shown that plants of these species grown in the greenhouse under the same temperature conditions during the winter in days lengthened by means of incandescent lamps were always much taller than plants grown in the normal or short day lengths. It is also noted that plants of *A. cristatum* and *A. smithii* bloomed only in the long days, and plants of *B. gracilis*—while they would bloom in either day length—were always slower to come into flower in the long day. Throughout these shading experiments similarities in growth form and blooming response were noted between plants growing in the reduced light intensities and in the artificially lengthened days of the winter and between plants growing in full sunlight and in the short days of the winter season. Whether or not these similarities are coincidental is not known.

Summary

1. Plants of *Agropyron cristatum*, *A. smithii*, and *Bouteloua gracilis* were grown at Cheyenne, Wyoming, in 100 per cent, 57 per cent, 42 per cent, and 28 per cent sunlight in 1938 and in 100 per cent, 70 per cent, and 50 per cent sunlight in 1939, and the height, dry weight, and number of plants that flowered recorded.

2. The plants grown in the shade were taller than those grown in full sunlight but had a smaller dry weight.

3. Reducing the light intensity to a certain extent increased the number of plants of *A. cristatum* and *A. smithii* which flowered but greatly decreased the number of plants of *B. gracilis* which flowered.

4. Using the dry weight as a measure of growth, the results indicate that these species make their best growth in full sunlight; but that full sunlight, while favoring the flowering of *B. gracilis*, is so intense as to retard the flowering of *A. cristatum* and *A. smithii*.

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USE OF FROZEN VACUUM-DRIED MATERIAL IN AUXIN AND OTHER CHEMICAL ANALYSES OF PLANT ORGANS: ITS EXTRACTION WITH DRY ETHER[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 522

GEORGE K. K. LINK, VIRGINIA EGGERS, AND JAMES E. MOULTON

(WITH ONE FIGURE)

Standardized and quantitative methods of extraction of auxins are greatly needed to foster studies of the presence and role of auxins in normal and healthy, and in abnormal and pathic, growth in plants.

In current practice each test proceeds from a sample of fresh plant material placed either in ether, alcohol, or water, alone or in a series of one or more of these solvents (3, 20). In general, grinding or even moderate division of the material is avoided to escape the destructive action of oxidative enzymes on the auxins. It is likely that the variation in chemical constitution known to occur in plant organs or tissues from hour to hour, day to day, and growth phase to growth phase, also holds for the auxin contents of different samples of the "same" material. It seemed advisable, therefore, to devise methods which permit collection at one moment of a large sample of fresh material which might then be drawn on for identical subsamples. Such a method would require a thoroughly homogeneous sample, which could be attained only by grinding to fine powder and careful sifting. It would eliminate one of the many variables which hamper current procedures of auxologists. AVERY (1, 2) has approached this method by using air-dry seed of corn, which he grinds before extraction. SKOOG and THIMANN (17) have used ground samples previously dried by heat.

Current procedure also is based on the report and assumption (14) that a single extraction of an intact plant organ with ether, alcohol, or chloroform quantitatively removes the auxins. LINSER (12) reports that this is not the case for spinach, and AVERY (2) reports the same for corn endosperm; GUSTAFSON (7) for tomato and other plants, and SKOOG and THIMANN (17) for *Lemna* and other plants. We have made the same observation for each of the materials under study: roots and nodules of kidney bean, soybean, garden pea; leaves of cabbage and the aphids *Myzus persicae* and *Brevicoryne brassicae* which feed on them; hypocotyl of tomato and the hypocotyledonary galls incited in it by experimental inoculation with *Phytomonas tumefaciens;* the leaves and tassels of corn as well as the smut galls

[1] This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. It also was aided by student help provided by the N.Y.A.

incited in them by experimental inoculation with *Ustilago zeae*, and the aphid *Aphis maidis* which feeds on them.[2] The findings suggest that the auxins are progressively liberated from plant tissues, and that water might play a role catalytic or otherwise in this process. It seemed advisable, therefore, to test the behavior of really dry materials in the presence of really dry solvents.

Current practice also proceeds from the observation (**19**) that oxidative enzymes of plants rapidly destroy auxins. For this reason fresh plant materials generally are placed as whole as possible into peroxide-free ether (**3**) or other solvents. This suggested that drying by heat should be avoided if possible. The alternative of arresting enzyme activity and breaking up the material by freezing, tried by DU BUY (**4**) in chlorophyll-containing tissues, has not come into general use because it is troublesome, and because—unless special care is taken to avoid thawing in air—the material is even more subject to destructive enzyme activity than before its injury by freezing.

Apparatus and methods

Seeking a method which would attain the objective of a large dry sample without much enzymatic and other chemical alteration, the senior writer recollected the class lectures given by Professor F. C. KOCH between 1910–1916 in a course on tissue analysis at the University of Chicago. In these he called attention to SHACKELL's (**16**) method of freezing animal tissues (solid and fluid) and then dehydrating them in vacuo, and pointed out that this would be the ideal method of preparing biological tissues for analysis, if the technical difficulties of rapid freezing and drying in vacuo could be mastered.

During a consultation with Professor KOCH in 1940 on this topic he called attention to the fact that the technical difficulties of freezing and dehydrating in vacuum had been overcome to such an extent that the method had come into commercial use by firms which process biological products, notably seric and endocrinic, from tissues. This advance is largely the result of the investigations of FLOSDORF and others working in collaboration with MUDD (**5, 6, 13**). Their papers contain a historical statement of the development of the process and many technical details about apparatus and techniques. TAYLOR and BEARD (**18**) recently described an inexpensive modification of FLOSDORF and MUDD's latest model (**6**) of the apparatus.

This method of preparing biological materials involves a minimum of chemical change in them. Only certain proteins and enzymes seem to be altered by the dehydration. So long as the material is solidly frozen, enzymatic activity is virtually absent. Since evaporation of the ice in the vacuum cools the material, this remains cold until dry, with the result that the enzymes are then inactive

[2] The work on corn, corn aphid, corn smut, and *Ustilago zeae* is being carried out by J. E. MOULTON.

because of lack of water. Unlike tissues dried by heat or chemical reagents, these avidly take up as much fluid as they lost, and hence are called lyophile.

By this method material of legume roots and nodules, of tomato hypocotyl and gall, of corn leaf, corn smut galls, and of the fungus *Ustilago zeae* has been prepared, using the apparatus of Professor KOCH and THOMAS F. GALLAGHER of the Department of Biochemistry. Based on these experiences and in consultations with these colleagues, to whom we are greatly indebted for many courtesies, a modification of their apparatus has been devised which is suited to the handling of plant materials, which usually are more bulky than animal tissues.

The apparatus differs from some others in current use in that the distillate from the frozen material is not adsorbed by a desiccant in a trap; instead it is collected in a freezing trap and is recoverable after thawing and thus available for analysis and experimental use. Experience with each of the plant materials prepared in the apparatus shows that the distillate is not pure water; it is slightly turbid and has the odor characteristic of the material dried. Drying by heat, or drying by the recent cryochem method of FLOSDORF and MUDD (**6**), apparently loses some of the constituents, and consequently a complete compositional picture of the material cannot be constructed from analysis of the dried residue. In all likelihood the activity of such material also is not the same as that of fresh material.

The apparatus (fig. 1) is made of heavy pyrex glass[3] with interchangeable ground-glass connections. Since the average free path of the molecules in the line is an important factor in the evacuation and drying, a large and uniform passage was attained by using 34-mm. (inside diameter) tubing with 40/50 interchangeable connections.

The essential parts are pear-shaped pyrex flasks (*A*) with 40/50 male connections. The material may be frozen directly in such flasks. The male connection avoids use of vacuum grease inside the neck of the flask and possible contamination of the sample. The connection of each flask fits into a 40/50 female connection at the end of one of two short arms which project at an angle of 80° from the upper part of the freezing trap (*B*). This trap is 100 mm. in diameter and 23 cm. long below the arms, with a capacity of 1800 cc. of fluid. The arms are set at an angle of 80° to give maximum surface exposure to the material in the pear-shaped flask (500 cc. or 1000 cc.). The larger the flask, the thinner the layer of a given amount of material, and hence the more rapid the drying. The arms, 50 mm. long, are as short as possible to minimize the path of the water molecules and just to clear the outer edge of the container (*C*), in which the trap is set when in use. Container *C* is a pyrex 1-gallon food jar (Jumbo).[4] After the trap (*B*) is sus-

[3] Made for this work by the Scientific Glass Apparatus Co. of Bloomfield, N.J.

[4] Jar no. 8642 or no. 820 of the American Thermos Bottle Co. is recommended. The former is more highly evacuated than the latter and is more expensive. We are using the latter (Jumbo) with inside dimension of $5\frac{7}{8} \times 11$ inches.

pended in this container, crushed dry ice (CO_2) and the methyl ether of ethylene glycol[5] are slowly packed around it up to the rim. This mixture, which is also used for freezing the material, gives temperatures between $-78°$ and $-80°$ C. It is less expensive and more pleasant to work with than acetone. Traps *B* and containers *C* can be set up in series up to the capacity of the pump system. If additional freezing traps are used, these (B_1, B_2, etc.) differ only in that each has an inlet arm (*i*) for connecting to the line. The large trap obviates interruption of the desiccation when large samples are being dried, and the large thermos container (*C*) reduces the time required in restoking with dry ice. A late evening stoking will carry the apparatus through the night and well into the late morning.

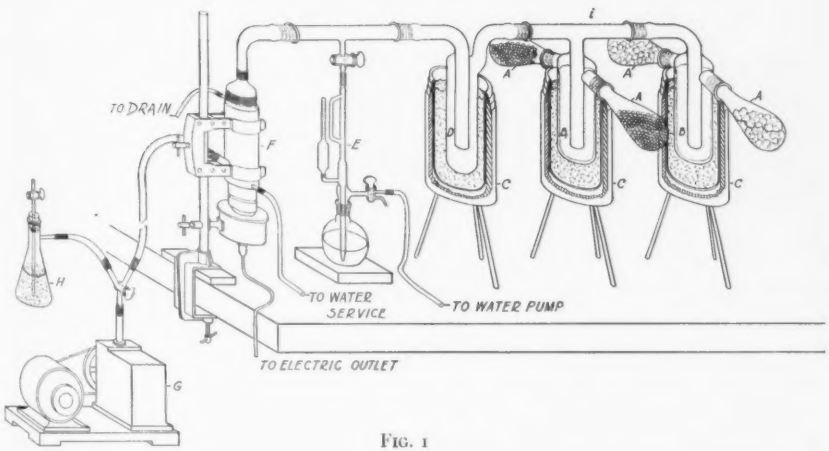

FIG. 1

Beyond the last side arm trap is a smaller safety freezing trap (*D*). This trap, 75 mm. in diameter and 23 cm. long, is set in a cellosolve dry-ice bath in a thermos jar. It ensures trapping of any water vapor which got by the other traps, and also protects these traps from the mercury of the gauge and pumps.

Beyond the safety trap (*D*) is a MacLeod mercury gauge (*E*) and beyond this a Cenco metal mercury vapor diffusion pump (Super Vac) (*F*) which is fitted to the 34-mm. pyrex line with an adapter by means of Cenco pure para rubber tape. This pump is capable of generating a vacuum of 10^{-6} mm. at a speed of 7 liters per second. Such is not attainable of course in a line containing water vapor. Beyond the mercury pump is a High Vac oil pump (*G*) which serves as forepump. For best results, the line between this and the Super Vac mercury pump consists of a pyrex glass rather than of rubber tubing. Air is admitted to the system through a chemical desiccant bottle (*H*) when the vacuum is to be broken. The maximum capacity

[5] Sold as Cellosolve by the Union Carbide and Carbon Co.

of the system has not been determined. Use of two traps, each with two 500-cc. flasks containing 50 gm. fresh bean material each, gave a dry product in 18 hours, with a total consumption for freezing and drying of 10 lb. of dry ice. Such samples are adequate for complete microchemical analysis. The dried material is readily ground in a Wiley Micro mill. The powder is ground to pass a 60 mesh and then stored in an evacuated desiccator over phosphoric pentoxide.

In case it is not essential to save the distillate, the apparatus described by TAYLOR and BEARD (**16**) can be modified by fitting the desiccant chamber with metal piping (four outlets at least) to which 34-mm. pyrex tubes (or any smaller size), each with an interchangeable female connection, can be fastened with the rubber tape. Into the female part may be fitted the flasks of any size with male 40/50 connections.

Experimental results

By use of material of fresh and of frozen-dried roots of red kidney bean, of fresh and of frozen-dried nodules, and of frozen-dried hypocotyl of tomato and of galls produced in this organ by inoculation with *Phytomonas tumefaciens*, a comparison has been made of the activity of the wet ether extracts of these materials. The results are given in table 1. It is evident from the table that the frozen-dried material yields more potent extracts, not only in the first but also in subsequent extractions. Incidentally the extracts of nodules are much more auxinic than are those of roots. These findings corroborate earlier reports (**8, 10**).

A study of the role of dry ether, absolute alcohol, and water in the extraction of auxins was made, using bean nodule and tomato gall material prepared by the freezing and drying method. For controls, equal weights of the dry powder of the nodules (0.175 gm. = 1 gm. wet weight) were extracted for 5 days with 50 cc. of ether (column 1) distilled over $FeSO_4$ and CaO in water (table 2). The other types of solvents are indicated in columns 2–9. Only the results for the nodule material are reported numerically. The ether used in columns 2–7 was distilled and stored over sodium to render it both anhydrous and free of peroxides. The water added was distilled, and boiled to remove oxygen, as was that used in column 8. The extract in each case was evaporated to dryness in a water bath at 45° C., except that of column 8 which was evaporated in the apparatus already described. This yielded a beautiful feathery residue which promptly took up water. Each residue was taken up in 2 cc. of 1.5 per cent agar. The first dilution tested therefore represents the ether extract from 0.5 gm. of wet material. The standard *Avena* coleoptile test was used, and the indoleacetic acid values for the test days are given. Since there is no unanimity among auxologists as to the unit to be used in recording the results of this test, and since all units proposed so far are subject to serious shortcomings, the data are reported without conversion into units, so that they may be used by anyone, once a unit is agreed upon.

Table 2 shows that the dry solvents, ether and alcohol, do not contain any active fractions after standing over the material for 5 days or after Soxhletization

TABLE 1

COMPARISON OF AUXINIC ACTIVITY OF WET ETHER EXTRACTS OF FRESH AND OF FROZEN VACUUM-DRIED NODULES AND ROOTS OF RED KIDNEY BEAN, EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES)

Material and treatment	Grams fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar	Testing dates										
		10/18	10/23	10/25	10/30	11/1	11/6	11/20	11/27	12/4	12/11	1/31
Nodules: fresh into wet ether 10/9/40 (1 vol. in 50)	0.0078	16.6	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0156	23.5	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0312	32.3	.....	0	.....	.....	.....	.....	.....	.....	.....	.....
	0.0625	29.9	.....	1.7	.....	.....	.....	.....	.....	.....	.....	.....
	0.1250	27.5	.....	9.3	.....	0	.....	.....	.....	.....	.....	.....
	0.2500	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....
	0.5000	.....	.....	.....	.....	2.2*	.....	.....	.....	.....	.....	.....
	1.0000	.....	.....	.....	.....	10.4	.....	.....	.....	.....	.....	.....
Roots: Fresh into wet ether 10/9/40 (1 vol. in 50)	1.25	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	2.5	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	5.0	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
	10.0	0	.....	0	.....	0	.....	.....	.....	.....	.....	.....
Nodules: Frozen and vacuum-dried 10/9/40, into wet ether 10/18/40 (1 vol. in 50)	0.00095	.....	1.0	.....	.....	.....	.....	.....	.....	.....	.....	.....
	0.0019	.....	3.4	.....	.....	.....	.....	.....	.....	.....	.....	.....
	0.0039	.....	8.4	.....	0.5	.....	.....	.....	.....	.....	.....	.....
	0.0078	.....	.....	.....	3.3	.....	0.7	.....	.....	.....	.....	.....
	0.0156	.....	21.5	.....	10.0	.....	1.9	.....	.....	.....	.....	.....
	0.0312	.....	.....	.....	18.9	.....	4.6	.....	.....	.....	.....	1.1
	0.0625	.....	23.6	.....	23.3	.....	10.5	.....	7.4	8.6	7.3	6.0
	0.1250	.....	.....	.....	28.8	.....	19.7	19.0	17.7	12.1	17.2	17.0
	0.2500	.....	18.4	.....	30.5	.....	23.6	21.2	22.4	20.7	23.3	23.9
	0.5000	.....	.....	.....	23.9	.....	20.1	21.7	23.8	25.0	32.0	29.5
	1.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Roots: Frozen and vacuum-dried 10/9/40 into wet ether 10/18/40 (1 vol. in 50)	1.25	.....	0.2	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2.5	.....	3.2	.....	6.4	.....	0.3	.....	.....	.....	.....	6.7
	5.0	.....	10.9	.....	12.2	.....	4.0	.....	.....	.....	2.3	12.7
	10.0	.....	13.6	.....	15.3	.....	9.9	9.2	18.0	17.2	6.8	19.2
Indoleacetic acid:												
20γ	.....	15.4	9.4	14.7	10.7	8.8	8.1	10.2	9.0	11.8	14.8	11.2
40γ	.....	24.6	14.7	22.9	20.7	20.1	14.5	15.1	21.9	16.8	19.0	25.9

* Discontinued for lack of treating time. In preceding experiment (not recorded) activity continued at this low level from seventh to eleventh extraction.

for 3–7½ hours. The same order of results, which will be published in another paper, was obtained with tomato hypocotyl and crown gall of tomato. The table also shows that when wet ether is used, or dry ether to which water has been added, the extract is active in the *Avena* test and water alone gives a highly active extract.

In another experiment dry bean nodule material was extracted for 5 days with 50 cc. of dry ether and another lot daily for 5 days with 10 cc. of dry ether per day, the residues being pooled for the test. The ether extracts of each sample were inactive. The extracted residues were divided each into two lots and used with other samples for some of the tests recorded in table 3. It was expected that the Soxhletized material would yield very active extracts and hence it was decided to test high dilutions. Because we were mistaken in this we just caught the upper range of activity of the extracts with the lowest dilutions. Identical results were

TABLE 2

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF RED KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES) (ETHER DRIED OVER SODIUM, EXCEPTING AS INDICATED)

GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	WET ETHER 50 CC., FOR 5 DAYS	DRY ETHER 50 CC., FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. H_2O, FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. 100% ALCOHOL, FOR 5 DAYS	DRY ETHER 50 CC. + 1 CC. 100% ALCOHOL + 1 CC. H_2O, FOR 5 DAYS	SOXHLETIZED FOR 3 HOURS WITH 240 CC. DRY ETHER	SOXHLETIZED FOR 3 HOURS WITH 240 CC. DRY ETHER + 1 CC. H_2O	50 CC. OF WATER, FOR 5 DAYS
0.00195	8.1	0	4.9	0	2.3	0	0	0.8
0.0078	31.5	0	25.3	0	14.8	0	9.6	12.5
0.0312	38.0	0	36.5	0	25.6	0	.....	25.3
0.125	23.0	0	24.2	0	26.9	0	.....	.....
0.50	6.5	0	13.7	0	14.1	0	.....	.....
Indoleacetic acid:								
20γ			17.4				11.0	
40γ			24.9				19.7	

obtained with dried material of tomato hypocotyl and tomato crown gall, except that the numerical values are different. These will be published later.

The results of table 3 show that previous extraction of dry material with dry ether does not affect its auxin content; in fact, this may facilitate subsequent extraction with water or with aqueous ether. The relatively low yields after Soxhletization with wet ether probably are due to the short time (3–7½ hours) during which wet ether was in contact with the material. That they are not due primarily to destruction of auxin by the temperature of boiling ether is evident from the table. Table 3 also shows that the dry ether extract does not contain a fraction which can be activated by addition of water. For a time this was thought to be the case and it was assumed that this meant that dry ether extracts contain an inactive precursor (such as an ester) which is rendered active by water. This was an

error. In not one of many subsequent tests was it possible to duplicate these results, although they were repeated with ether residues of the lot from which they had been obtained. It was concluded that the original extraction was not carried out with dry ether, as previously thought, and that the thimble of the Soxhlet which was washed with wet ether had not been allowed to dry sufficiently. This suggests that small amounts of water in ether suffice to give an active extract. The minimal amount necessary has not been determined, nor the optimal time for extraction. Both nodule and tomato material show that a 120-hour extraction

TABLE 3

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF RED KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES). MATERIAL PREVIOUSLY EXTRACTED WITH DRY ETHER FOR TESTS OF TABLE 2 (ETHER DRIED OVER SODIUM)

GRAMS FRESH MATERIAL EXTRACTED REPRESENTED BY ITS PORTION OF EXTRACT IN 1 CC. OF 1.5% AGAR	RE-EXTRACTED FOR 5 DAYS WITH 50 CC. DRY ETHER+1 CC. H_2O	RE-EXTRACTED FOR 5 DAYS WITH 50 CC. DRY ETHER TO WHICH 1 CC. H_2O WAS ADDED AFTER EXTRACTION	RE-EXTRACTED WITH 50 CC. DRY ETHER+1 CC. H_2O	
			HALF REFLUXED FOR 3½ HOURS	HALF NOT REFLUXED
0.00048	.....	0	0	0
0.00097	.....	.....	0	0
0.00195	11.7	0	0	0
0.0039	22.3	.....	3.3	2.6
0.0078	36.5	0	6.7	7.6
0.0156	.....	.....	.....	.....
0.031	.....	0	.....	.....
Indoleacetic acid:				
20γ			11.0	
40γ			19.7	

with 50 cc. of wet ether gives better results than a 24-hour extraction, or than a 7½-hour extraction in a Soxhlet with 1250 cc. of ether-water passing over the material. No complete study has been made of the amount of ether best suited for maximal extraction, nor of the role of temperature.

One extraction of dry material with wet ether does not remove all auxin (table 1). The dry powder avidly takes up the water from the solvents, swells, and becomes colloidal. The number of extractions necessary for its complete removal has not been determined (table 1). This applies both to nodule and to tomato materials. The combined extracts of a dry ether extraction and of a subsequent wet one yield a highly active preparation which, however, is neither more nor less active than the wet ether extract alone (table 4); that is, there is no evidence of synergism. The same applies to extracts of tomato gall.

Evaporation of ether-water extracts, and subsequent treatment of the residue (dried in an Abderhalden vacuum dryer[6]) with dry ether yields an active preparation (table 4). The same result was obtained with extracts of the tomato gall.

TABLE 4

AUXINIC ACTIVITY OF ETHER EXTRACTS OF FROZEN VACUUM-DRIED NODULES OF KIDNEY BEAN EXPRESSED IN AVERAGE CURVATURE PER TWELVE AVENA COLEOPTILES (DEGREES) (ETHER DRIED OVER SODIUM)

Grams fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar	Extracted 3 days with 50 cc. dry ether; extract evaporated to dryness at 45° C.			Extracted 1 day with 50 cc. dry ether; filtrate evaporated and residue stored (A); powder re-extracted 5 days with 50 cc. dry ether and 1 cc. H_2O; extract evaporated, residue (B) combined with (A), and taken up in agar
	Residue taken up in agar	Powder re-extracted 5 days with 50 cc. ether+1 cc. H_2O; extract evaporated, residue dried in vacuum, then treated with dry ether		
		Solution taken up in agar	Residue taken up in H_2O and agar	
0.000195	0.0	0.0	0.0	0.0
0.00039	0.0	1.1	0.0	0.0
0.00078	0.0	5.0	0.0	7.2
0.00157	0.0	15.6	0.0	19.0
0.00315	0.0	24.0	0.0	25.8
0.0062	0.0	27.1	0.0	23.8
0.0125	0.0	22.3	2.0	26.4
0.025	0.0	.....	8.1	24.3
0.050	0.0	.....	15.3	.....
Indoleacetic acid:				
20γ	10.0		11.2	13.3
40γ	18.6		25.9	18.7

Discussion

These results indicate that the method of extracting auxins from frozen vacuum-dried material is superior to extraction of fresh material. While the fresh roots yielded an inactive extract, the frozen-dried roots gave an active extract. Also the yields obtained from frozen-dried nodules are higher than from fresh nodules. The results also indicate that water plays some role in liberating the active fraction of ether extracts of auxin from plant tissues, and that this fraction is soluble in dry ether. The findings of SKOOG and THIMANN (**17**) indicate that this is done hydrolytically, and that proteolytic enzymes, such as chymotrypsin, may function in the process. Our results indicate that any free auxin present in living tissues either is destroyed in the process of freezing or of dehydration, or of both,

[6] We are indebted to Dr. T. F. GALLAGHER for use of this apparatus.

or is fixed during these processes so that none is removed by dry ether. Possibly this may be correlated with the fact that dry tissues do not grow, that maturation involves dehydration, and that storage organs such as seeds do not yield auxins until water is added. If this is the case, then liberation and fixation of auxins in the plant are under metabolic control, and auxin itself is an agent whereby the plant controls some aspects of its growth, but is not the something which exercises that control.[7]

It is probable also that some of the difficulties of auxologists may have been due to the method of extracting whole fresh material by placing it in wet or in dry ether. The VAN OVERBEEK method (**14**) discards the water-ether mixture, which settles out if enough water is present. Much auxin may be lost by this procedure. Also the placing of fresh material in ether probably does not arrest all enzyme activity. Thus different amounts of auxin may be lost in different extractions, resulting in irregular or in negative results.

It seems probable that the difficulties others (**11, 15**) and we (**9**) have experienced in supporting the early findings (**9**) to the effect that crown-gall tissue of tomato contains more auxin than the normal tissue is in part due to these factors. In a later publication it will be shown that this difference does exist, as it does for all gall tissues tested by us to date. The results corroborate earlier reports (**8, 10**) that the nodules of the red kidney bean are much more auxinic than the roots.

If it should prove generally applicable that dry plant tissues do not yield auxin to dry ether, then this solvent may be used partially to defat and decolorize (dry ether removes chlorophyll completely) samples preceding extraction for auxins with water or watery solvents. This may prove a step toward isolation, identification, and quantitative estimate of auxins in plants. The dry material, prepared by freezing and drying in vacuo, should also prove highly useful in other chemical analyses of plant tissues.

Summary

1. A method is described for drying frozen plant material in vacuum with a minimum of chemical alteration, which is highly desirable for auxin and other chemical analyses. Material so prepared is auxinic and gives greater yields of auxins than fresh material.

2. Ether dried over sodium does not extract auxins from the material, nor does absolute alcohol. If a little water is added to such ether or to a dry ether-alcohol mixture, an active extract is obtained. Water extracts of such material alone are highly active. The material is lyophile, speedily taking up water from the solvent.

[7] After this paper had gone to press, a highly pertinent paper appeared: THIMANN, K. V., and SKOOG, F., The extraction of auxin from plant tissues. Amer. Jour. Bot. **27**:951–960. 1940. They, too, find that water is necessary for auxin extraction from dry material and that drying "fixes" the "unbound" auxin. They dried the material by heat and noted a decrease in yield of auxin due to heating.

3. The auxin liberated by water from dry material is soluble in dry ether. Water is necessary for the presence of active auxin in plant tissues. Freezing or drying, or both, apparently leads to destruction of active auxin or to its fixation in a nonremovable form.

4. Pretreatment of dry material with dry ether removes ether-soluble substances and yields a partially fat-free and ether-soluble pigment-free sample for auxin extraction. This should facilitate isolation and identification of auxins.

5. These findings so far apply to all materials tested: nodules of red kidney bean, hypocotyl of tomato, and hypocotyledonary gall of tomato incited by *Phytomonas tumefaciens*.

UNIVERSITY OF CHICAGO

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HISTOLOGICAL REACTIONS OF BEAN PLANTS TO L-TRYPTOPHANE

E. J. KRAUS[1]

(WITH FIFTEEN FIGURES)

Introduction

This paper is one of a series which deals with the histological reactions of plants to various growth-regulating substances. The l-tryptophane used was secured through the Eastman Kodak Company and was applied in the usual manner as a 2 per cent mixture with anhydrous lanolin. The details of procedure were the same as given earlier (3). Only one application was made. With other lots of bean plants it has been determined that a 0.5 per cent mixture is approximately as effective as the 2 per cent, and that the histological responses are similar. As in the case of the other growth substances so far worked with in detail, a more rapid and pronounced response occurs at a relatively high humidity than at a lower one, but in the experiments here reported the humidity was always well below saturation and no attempt was made to raise it in any way.

The strain of beans used was the same as in previous experiments. These plants were grown in benches filled with fertile potting soil in the greenhouses at the U.S. Horticultural Station at Beltsville, Maryland, during the spring of 1939. The actual period of the experiment from which the material illustrated was taken extended from May 1 to July 1. The usual care and attention as to watering and other details of growing were given. The temperature ranged from 18° to as high as 30° C. for short intervals on the brightest days. For the most part there was an abundance of sunshine. The control plants made what would commonly be referred to as excellent growth, were sturdy, and remained free from fungus, bacterial, or insect infestation. The specimens were preserved in Navashin's solution, handled according to the butyl-alcohol paraffin method and sectioned at 12 μ.

Gross responses to treatment

For the first 72 hours after treatment there is little external change except a slight paling of color near the treated surface; then enlargement takes place very rapidly. The effects are less noticeable 1–2 mm. below the surface. By the end

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of the sixth day a tumor 8–10 mm. in diameter may be formed (fig. 1). This is smooth, glistening, and yellowish white, with a distinct and abrupt depression at the center owing to lack of proliferation of the cells of the pith. Growth continues rapidly for 2–3 weeks, the tumor frequently becoming 2 cm. in diameter by this time and somewhat more dome-shaped, though never with a high crown or taper,

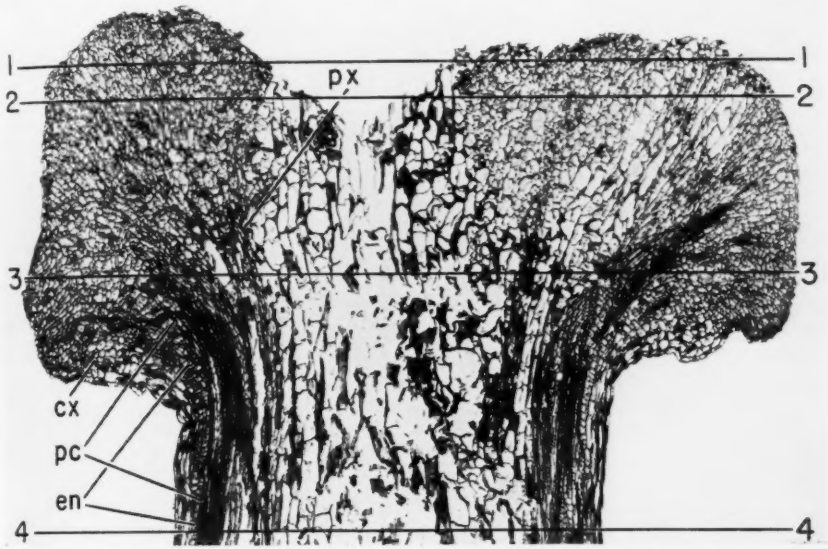

FIG. 1.—Median longisection of apical tumor 6 days after application of tryptophane-lanolin mixture: *cx*, cortical parenchyma cells only slightly divided and forced outward and downward by more actively meristematic cells centrad to them; *pc*, pericycle cells affected only slightly; *en*, greatly proliferated endodermis from whose derivatives, especially near the top, a vascular strand is being organized; *px*, primary xylem. Ends of vessels mark original surface of application. Younger parenchymatous cells of xylem, cambium, and phloem have proliferated markedly and form main portion of tumor, other than endodermal derivatives. Pith cells enlarged somewhat, then died, and became torn and disintegrated. Levels 1, 2, 3, 4 are referred to in connection with description of subsequent figures.

as in the case of those resulting from indoleacetic acid (2). Its surface is finely tuberculated, and the central depression is filled by the growth of peripheral tissues into it. The color becomes a dull gray, sometimes slightly greenish, owing to the death and suberization of some of the outermost cells and the differentiation of chloroplasts in others. After the fourth week growth proceeds slowly. The largest tumor observed at the end of 8 weeks was 2.5 cm.; most of them were appreciably smaller. Frequently large rifts occur over the upper surfaces of the tumors, owing to the death and drying out of cells. Of several hundreds of such

tumors examined, none was found with any roots or root primordia among its tissues or developing from the stem immediately below it. The tumor consists of parenchymatous cells and an intricate anastomosis of vascular strands of varying size, most of which are ultimately connected with the vascular bundles of the stem below it. A few short vascular strands are differentiated which remain completely surrounded by parenchymatous cells and are not directly connected to other vascular strands.

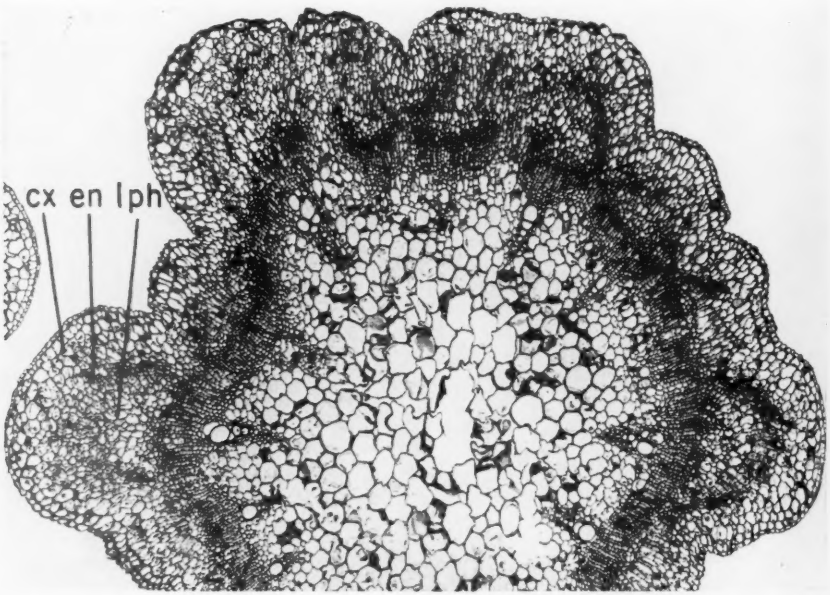

FIG. 2.—Section 1 mm. below treated surface 4 days after treatment. At left cortical parenchyma (*cx*) and endodermal cells are active, pericycle and phloem (ph_1) scarcely so, and pith has begun to disintegrate. At extreme right the endodermis proliferated, vascular bundle being organized near the rays, which are meristematic. Cambium shows varying degrees of activity.

Histological details

The epidermal cells in close contact with the lanolin-tryptophane mixture enlarge, frequently divide radially but do not proliferate greatly, so that they are generally ruptured by the end of 96 hours by the expanding tissues within them. Close to the surface of application the cells of the cortical parenchyma proliferate somewhat abundantly within 30 hours after treatment, and in some instances their derivatives may constitute a small portion of the apical tumors developed.

The endodermis is very sensitive in its response. Within 20 hours after treatment, and near the treated surface, the cells elongate radially and tangential di-

visions may occur. Subsequently enlargement and division take place with great rapidity, so that at first—in any given plane—a broad arc of proliferating cells is found over each main vascular bundle, with the activity somewhat less over the rays. Soon this proliferating mass of cells pushes the cortical cells ahead of it and

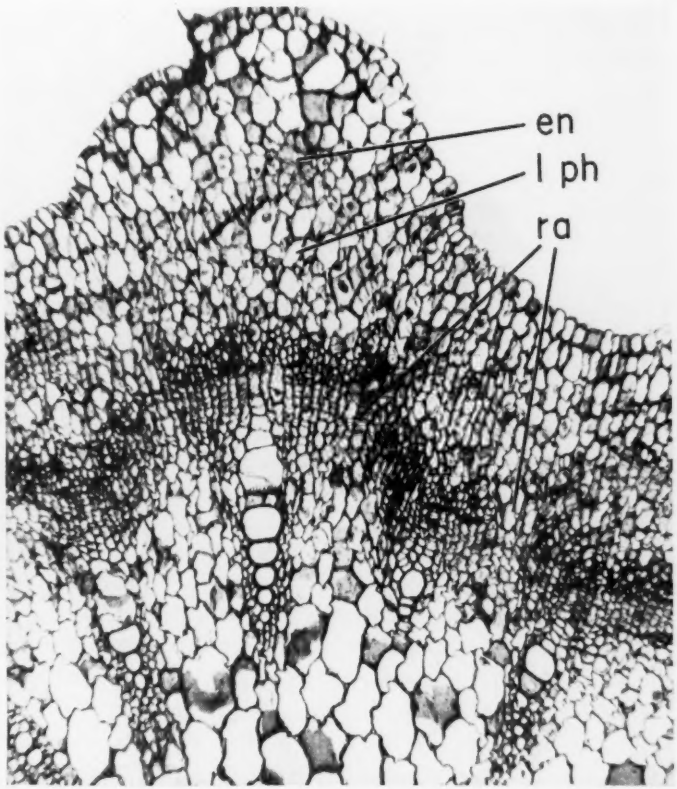

FIG. 3.—Single bundle similar to those in fig. 3, 0.5 mm. below treated surface, 3 days after application: *en*, endodermis; *ph*, phloem showing little effect; *ra*, one ray showing beginning of interfasicular cambium, other to right not yet active.

ruptures the epidermis. Throughout the mass, bands and groups of cells become differentiated as xylem and phloem tissues, with a more or less active cambium between them. These vascular strands anastomose with one another and are ultimately connected with the xylem and phloem of the stele (fig. 10). Farther down the stem, for distances of 4–5 mm. or more, the endodermis is active, progressively less so away from the treated surface. At the upper levels (figs. 2, 3) only those

endodermal cells over the apex of the primary phloem of a vascular bundle behave in a manner similar to that just described; those at the sides or margins proliferate to a less extent and give rise to vascular strands, the cambiums of which become connected with that of the stele through the progressive activity and differentiation of the cells of the medullary ray (figs. 7, 9, 10, 15).

Except immediately below the surface of application, the pericyclic cells show little response to the treatment.

The primary phloem, and to a less extent the secondary phloem, are highly reactive immediately below the treated surface, and within 72 hours after treatment (fig. 7) many derivative cells are formed. Some of these continue proliferation over a long period of time, others enlarge greatly into giant cells which may often be multinucleate, others collapse and die, and still others differentiate as vascular strands or as scattered groups of reticulate and wound tracheids (figs. 4, 11). About 0.5 mm. below the treated surface the primary phloem is active in much the same way as described, but the phloem parenchyma between the groups of sieve tube and companion cells is much less so. The type of subsequent development is similar to that at the higher levels but less extensive, although eventually a complex anastomosis of vascular strands results (figs. 12–14). At distances 2 mm. below the treated surface the cellular proliferation is less pronounced but may be appreciable by the end of 10 days (fig. 5*A*); at 4 mm. below there is little change in type of development, in so far as the phloem is concerned, from that of an undecapitated stem (fig. 5*B*).

The rays proliferate extensively at levels nearest the treated surface, and their derivatives differentiate much as do those of the phloem (figs. 4, 12, 13, 14). At slightly lower levels they are less active, and some of their derivatives form the cambium from which the vascular strands are developed between the endodermis and the vascular structures of the stele (figs. 6, 9, 15). In no case was the primordium of a root observed to arise from derivatives of the ray.

The cambium is highly sensitive near the treated surface, and after 12 hours cells derived from it divide in all planes and give rise to large masses of parenchymatous cells, some of which become extremely large and may have one to several nuclei; others continue active division, but the greater number of them mature as phloem or xylem elements, more particularly as reticulate tracheids in platelike masses and strands, and as vessels (figs. 8, 13, 14). At slightly lower levels the reaction is similar but much less marked, and at 2 mm. or more away the effects are still less obvious.

The xylem parenchyma near the treated surface is reactive in much the same manner as is the cambium. The very young vessel segments, which still possess nuclei and considerable cytoplasm, enlarge greatly, becoming enormous by comparison with the cells neighboring them (figs. 7*A*, 8). Frequently they divide. Some-

times they may contain several nuclei, but they usually collapse and disintegrate comparatively early in the development of the tumor. There is but little activity 1.5 mm. below the treated surface.

The pith cells respond but slightly to the treatment. Even at the treated surface those at the center of the stem do little more than enlarge for a time, then die and disintegrate (figs. 1, 4, 7). But opposite and adjacent to the points of the protoxylem of some of the vascular strands they may proliferate, continuing to do so for some days, finally wholly or in part filling in the cavity at the center of the stem resulting from disintegration of the pith and also the space above it which results from upgrowth of the cells of the peripheral tissues (figs. 1, 4, 11, 12). Only in the oldest tumors were a few traces of vascular bundles found in the tissues derived from the pith, but wound and reticulate tracheids were commonly differentiated.

Other details of the progressive development of the tumors and the several tissues which compose them are shown in the photomicrographs and the detailed descriptions which accompany them.

Discussion

The tissue responses of the bean stem to application of tryptophane in the development of tumors in some respects are similar to, and in many others quite different from, the responses to indoleacetic acid or any of the other compounds so far recorded in detail (**1, 2, 3, 4, 14**). The lack of initiation of root primordia, the differentiation of large numbers of vascular bundles (particularly those derived from the endodermis), and the precise method of development of their connection with the phloem and xylem of the bundles of the stele are particularly striking features, as are also the relative lack of responsiveness of the cells of the pith, whereas many of the cortical parenchyma cells are definitely sensitive.

The application of tryptophane to several species of plants and the subsequent proliferation of tissues and formation of gall-like growths has been cited by several investigators (**5, 6, 15**), but no detailed accounts have been found. WENT and THIMANN (**19**) state that tryptophane has no auxin activity but does accelerate the growth of *Avena* coleoptiles when applied to their base, that although inactive in itself it is subsequently readily converted to the active form by plant enzymes. This assumption is deemed probable by them because tryptophane may be converted to indoleacetic acid by fungi and bacteria. Whatever the value of such assumption, in so far as it may apply in relation to *Avena* coleoptiles, the histological responses of the bean stem to tryptophane are so different from those to indoleacetic acid, even in high dilution (**2, 3, 16**), that there seems no justification for making it in the latter instance. As yet there is available no direct evidence

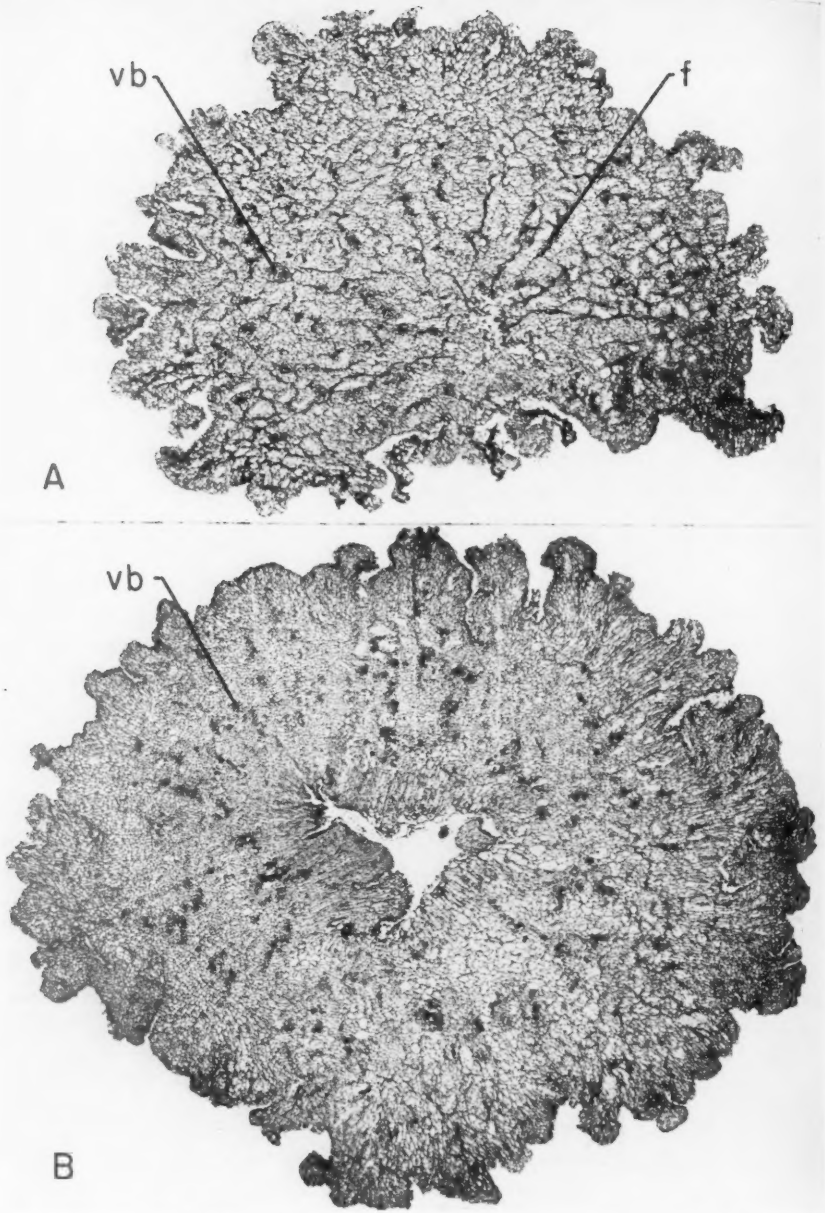

FIG. 4.—Transections of tumor 18 days after treatment. *A*, 0.5 mm. below upper surface (approximately level 1, fig. 1): *vb*, vascular bundles differentiated from proliferated tissues; *f*, folds and strands of collapsed, dead parenchymatous cells. Between these bundles and folds are active parenchymatous cells (note particularly margins of section). No appreciable cavity at center over dead pith, this having been filled in by tissues from cells derived from xylem, cambium, and phloem. *B*, same, 0.5 mm. below section in *A*: *vb*, vascular strands, and between them active parenchymatous cells from which additional bundles are being differentiated. Cavity at center is above dead pith and being filled in. Both sections are through proliferated tissues which have pushed up above surface of application.

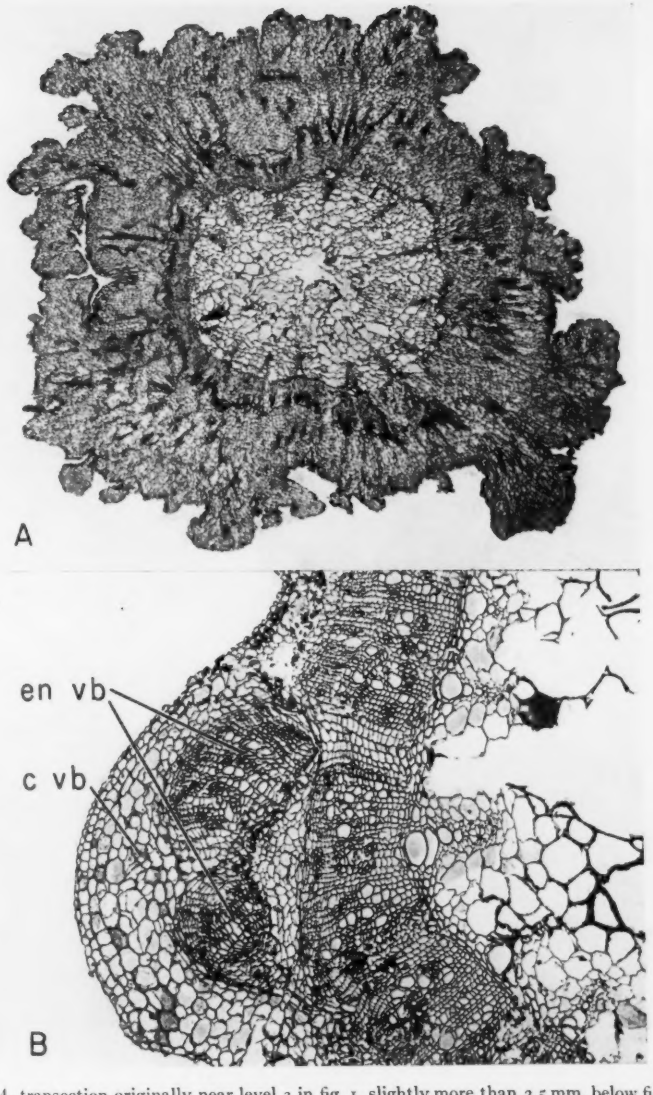

FIG. 5.—*A*, transection originally near level 3 in fig. 1, slightly more than 2.5 mm. below fig. 4*B* and below treated surface. At center is disintegrating pith, some of whose cells (opposite primary xylem) are proliferating and pushing into central cavity. Xylem already differentiated at time of application is evident. Cambium was markedly stimulated and its derivatives have matured into parenchymatous cells, tracheids of several types, tracheal segments, and at places complete vascular strands, each with separate cambium. Phloem parenchyma proliferated, and from its derivatives and those of endodermis, strands of sieve tubes and companion cells, a complex system of anastomosing vascular strands, and parenchymatous cells have been differentiated. *B*, same tumor, slightly below level 4, fig. 1. Pith disintegrated; xylem, cambium, phloem, and pericycle have developed as if untreated; from endodermal derivatives two large vascular bundles have differentiated, a small one forming from derivatives of cortical parenchyma. Somewhat below this level the endodermal bundles are directly connected with stelar bundles through vascular strands differentiated from derivatives of rays (cf. fig. 9*B*).

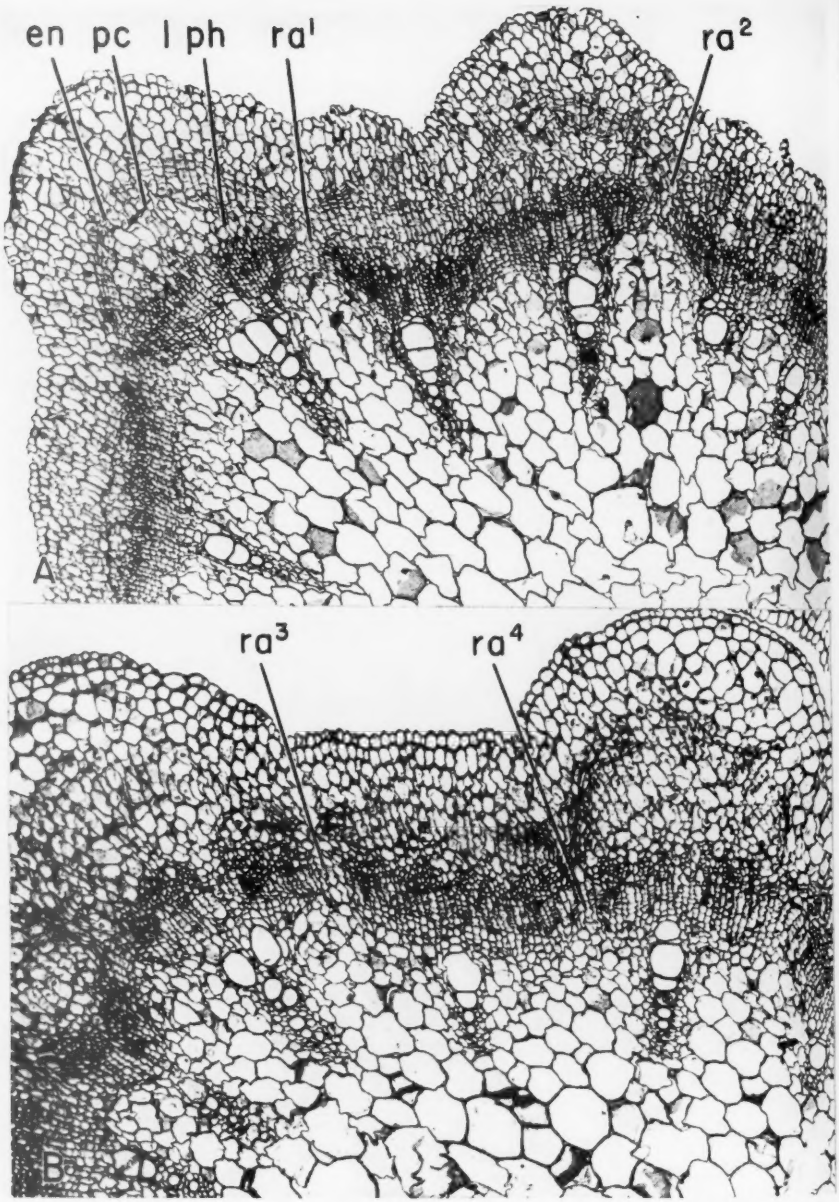

FIG. 6.—*A*, transection of 3-day-old tumor, 0.5 mm. below treated surface: *en*, active endodermis; *pc*, crushed pericycle; ph_1, slightly active primary phloem parenchyma; ra_1, activity beginning in ray above interfascicular cambium (cf. fig. 15*B*). *B*, similar to *A*, 4 days old. ra_3, ra_4, without and with interfascicular cambium.

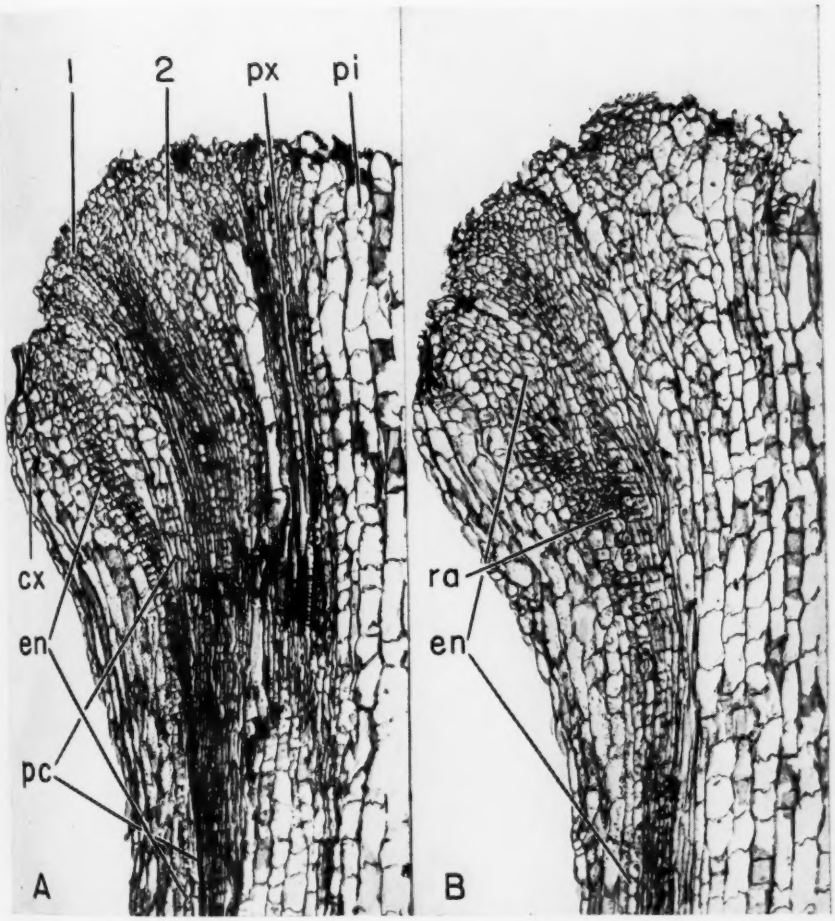

FIG. 7.—Longisection of tumor 4 days old. *A*, through midportion of bundle: *cx*, cortical parenchyma; *en*, endodermis; *pc*, pericycle; *px*, primary xylem; *pi*, pith. Except at extreme left, proliferating tissues have been considerably elevated above surface of application, indicated by dead and collapsed epidermis just above active cortical cells: *1*, cells derived mainly from phloem and phloem side of cambium; *2*, from xylem and xylem side. Large cells just left of primary xylem potential tracheal segments of secondary xylem. *B*, section through ray: *en*, endodermis; *ra*, proliferating ray cells.

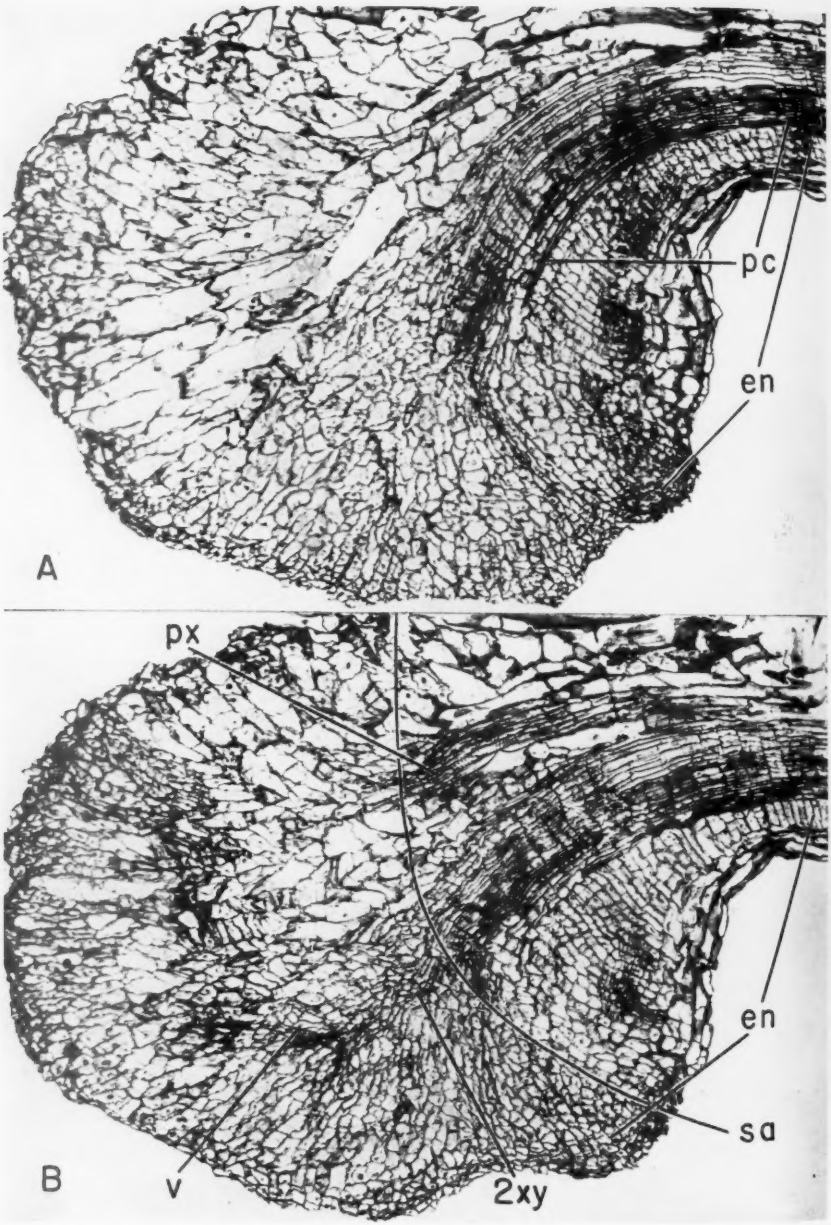

FIG. 8.—*A*, longisection of tumor 7 days old: *en*, endodermis; *pc*, pericycle. Portions of tumor derived from principal regions of stem readily distinguishable. Giant cells more conspicuous than in fig. 7. *B*, same tumor: *px*, primary xylem; *2 xy*, secondary xylem showing tracheae and tracheids; *v*, vascular bundle differentiating from derivatives of proliferated xylem, others from cambium and phloem regions at lower center; large lobe of tissue derived mainly from endodermis; few derivatives of cortical parenchyma. Surface of application (*sa*) now appears curved because of differential growth of tissues.

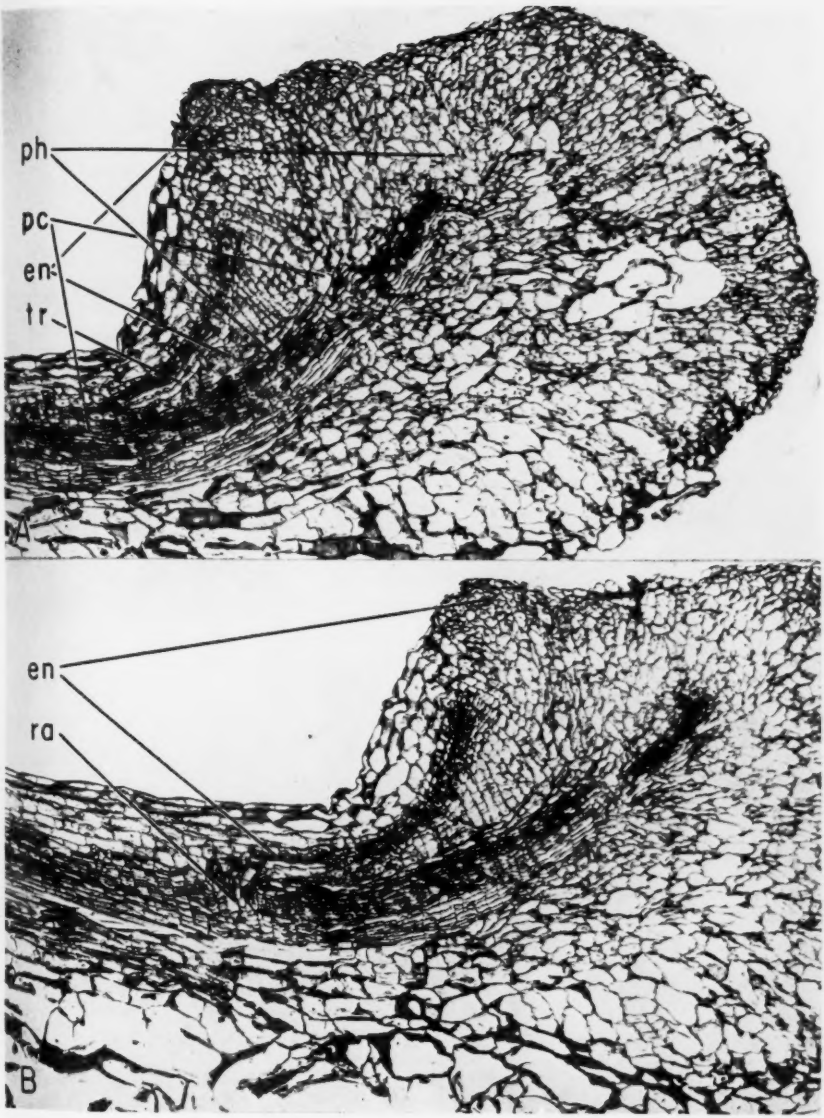

FIG. 9.—Same tumor as in fig. 8. *A*, vascular strand differentiated from endodermal derivatives: *tr*, tracheal segments and reticulate tracheids. *B*, connection of elements of vascular strand with those of stele through derivatives of ray (*ra*).

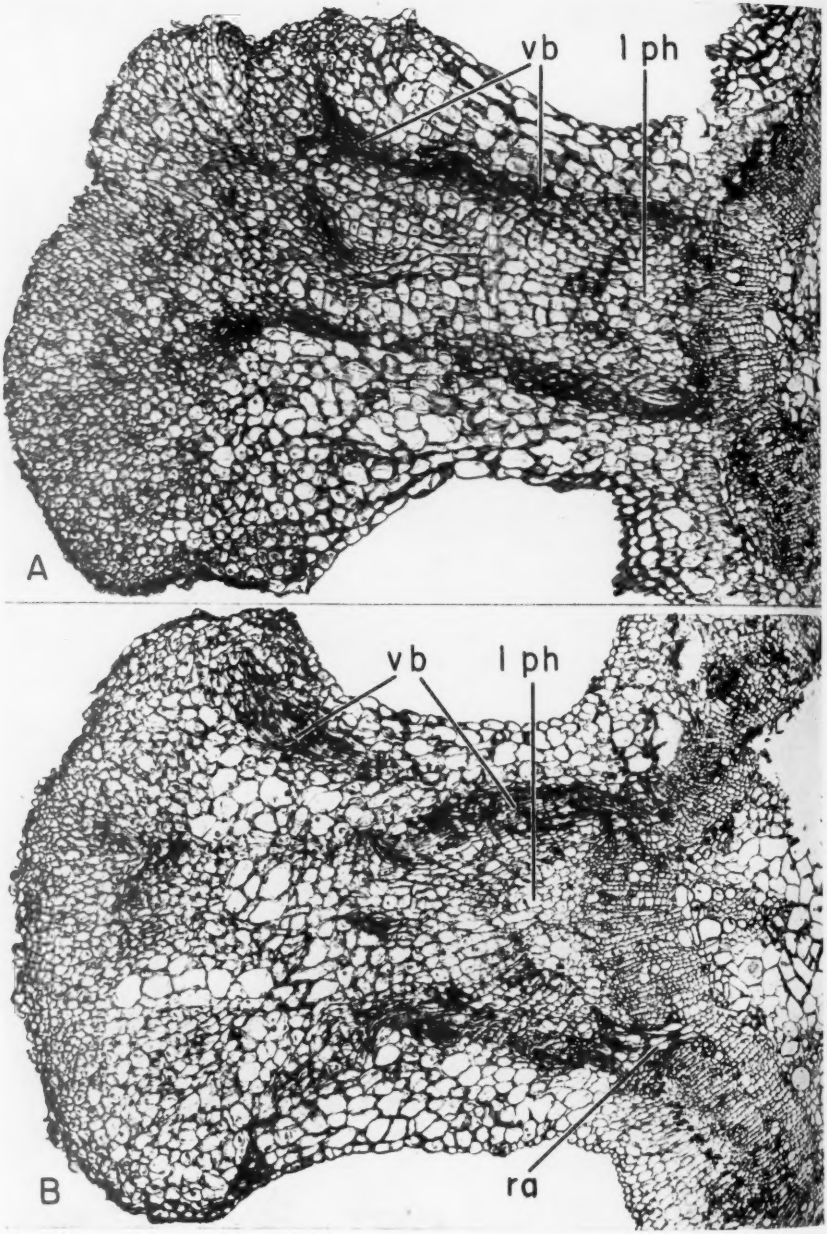

FIG. 10.—Sections through lateral projections of 7-day-old tumor near its lower margins. *A*, 1.75 mm. below upper surface. Most tissue involved derived from endodermis. Primary phloem (*ph*) slightly meristematic; pericyclic fibers pushed out of usual position but matured; other tissues centrad to these have matured as if untreated, except rays and their derivatives, from which have differentiated vascular elements connecting vascular strands (*vb*) of outgrowth with those of stele. *B*, similar section 2.5 mm. below upper surface. Most of this outgrowth is from endodermis and a few cells of cortical parenchyma which have pushed aside other cortical cells and ruptured epidermis (cf. fig. 5*B*).

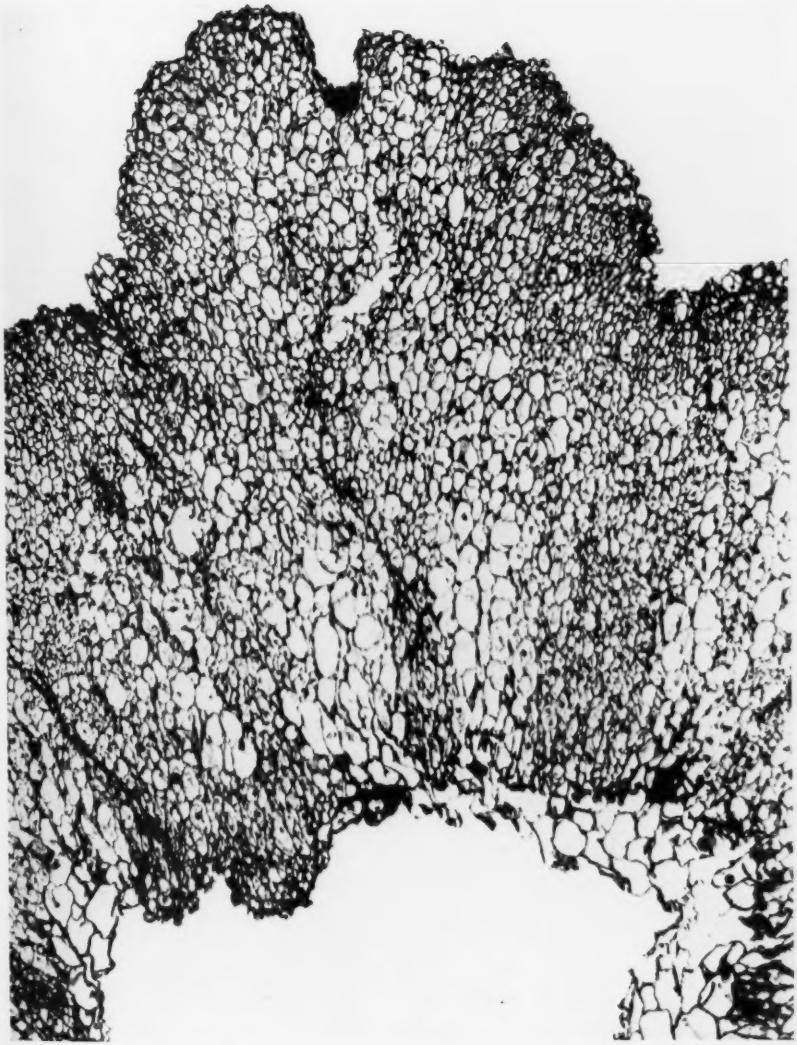

FIG. 11.—Transection of 6-day-old tumor 1.5 mm. below upper surface which has been elevated above level of surface of application (cf. fig. 8). Tissues derived mainly from phloem, cambium, xylem, and rays. At extreme bottom is cavity above pith. Dark patches and streaks are groups of sieve tubes and companion cells, and scattered here and there are tracheids, also giant cells which were potential tracheal segments, whose walls are too thin to be distinguished in photograph from associated parenchymatous cells. Groups of cells protruding into central cavity are mainly opposite and above points of protoxylem. The tear is characteristic; at later stages large holes and folds of dead collapsed cells develop.

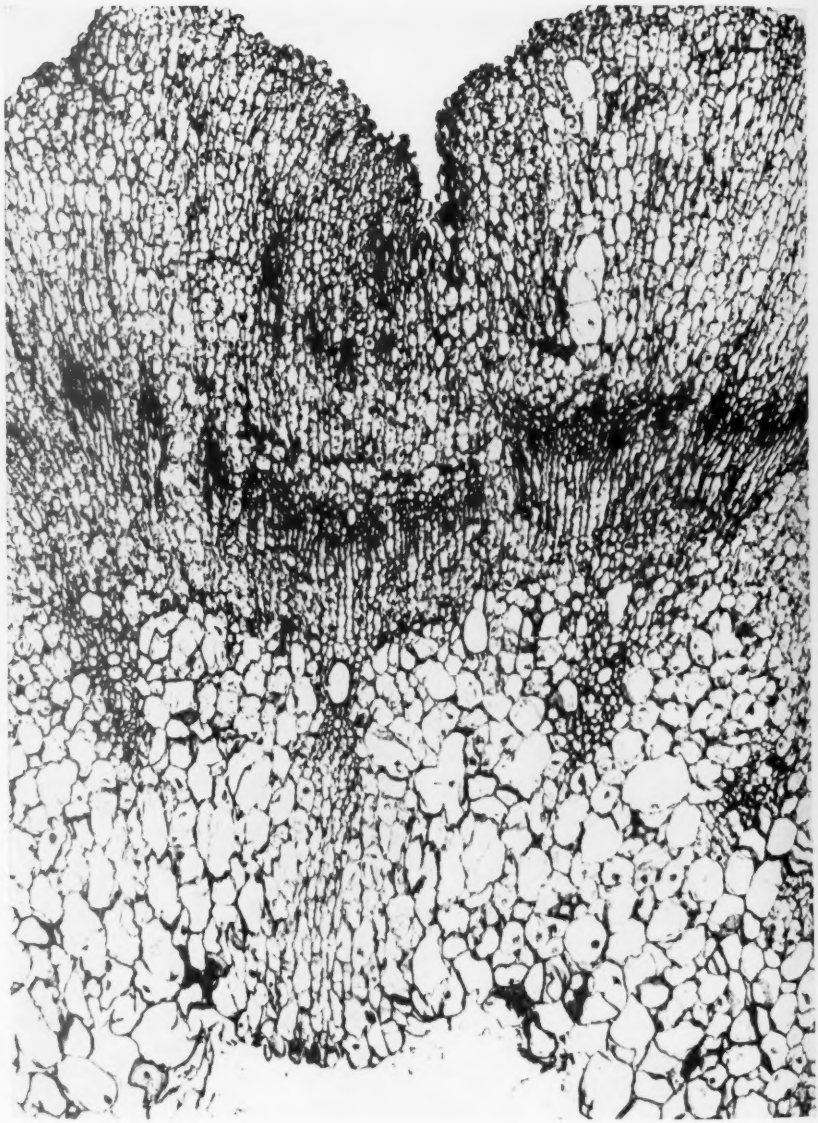

FIG. 12.—Same tumor as fig. 11, approximately 2 mm. below upper surface and just slightly below level of original surface of treatment. Pith cells are collapsed at center but somewhat meristematic opposite some points of primary xylem; cambium very active, its derivatives maturing into both phloem and xylem elements, many of which appear in semilongitudinal view because of upward thrust of tissues. Ray cells greatly enlarged and divided to some extent. Primary phloem parenchyma and endodermis have divided rapidly; some giant cells which generally collapse and die; other derivatives mature as vascular elements; still others continue rapid division and give rise to additional vascular strands and parenchyma. There are scattered tracheids, most of them reticulate; true vessels are numerous in vascular strands differentiated from proliferated tissues.

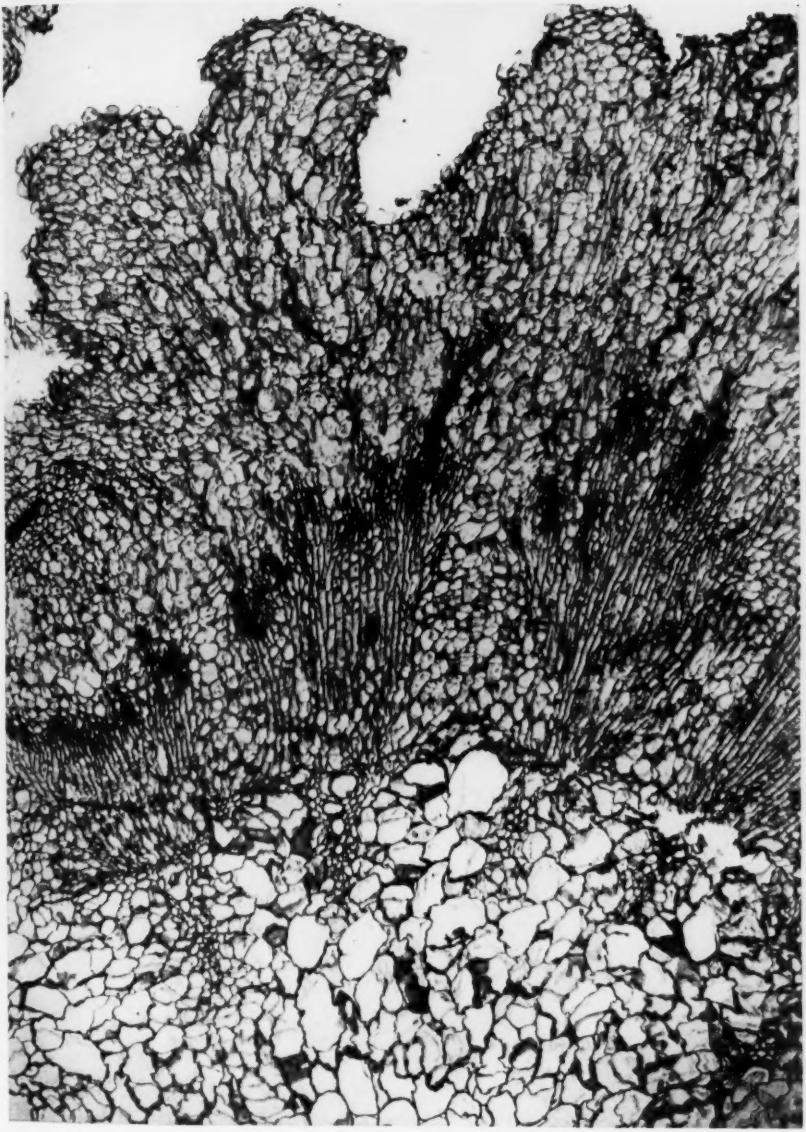

FIG. 13.—Transection of tumor 11 days old, about 2.5 mm. below upper surface and below original treated surface. Details similar to fig. 12. Tissues more clearly defined.

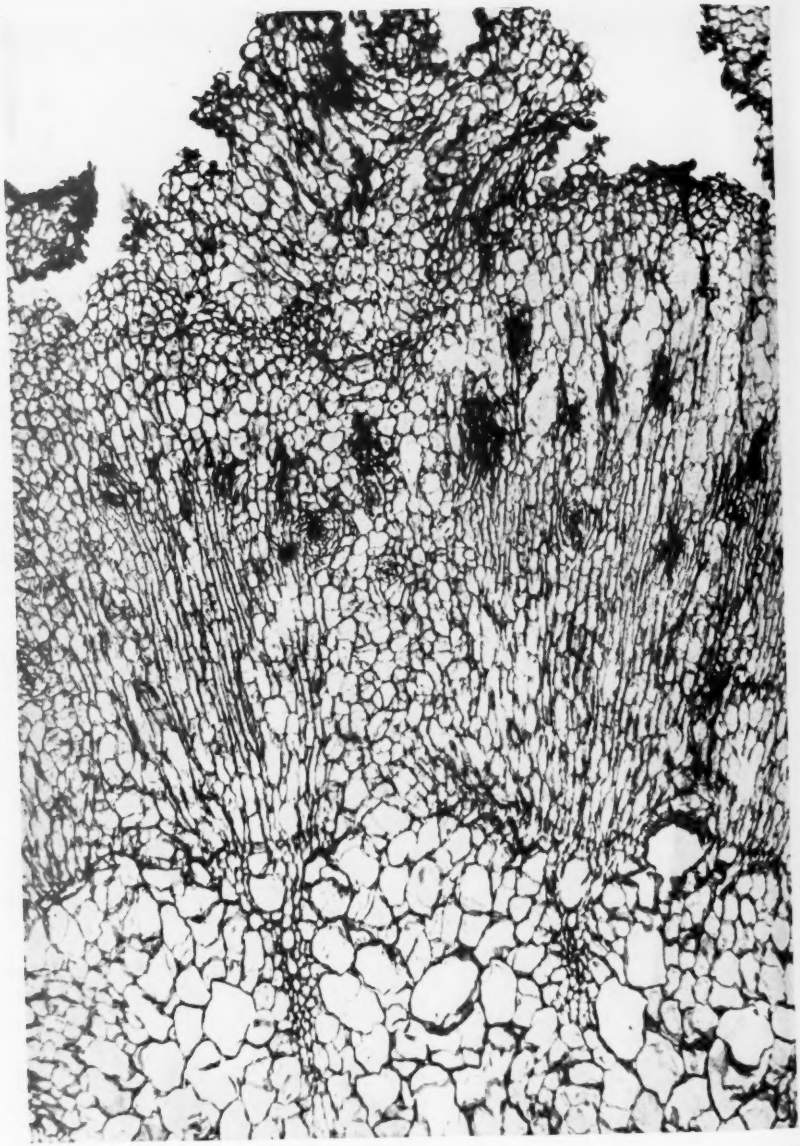

FIG. 14.—Same tumor as fig. 13 at slightly higher level, showing result of greater degree of proliferation of cambium and phloem with subsequent differentiation of anastomosing vascular strands and their relation to xylem of stele. Just below this level the appearance would be more nearly as in fig. 13, indicating connections of phloem. There is even greater variation in intricacy and pattern of differentiation at higher levels, but almost entire vascular network is directly or indirectly connected to vascular structure of stele.

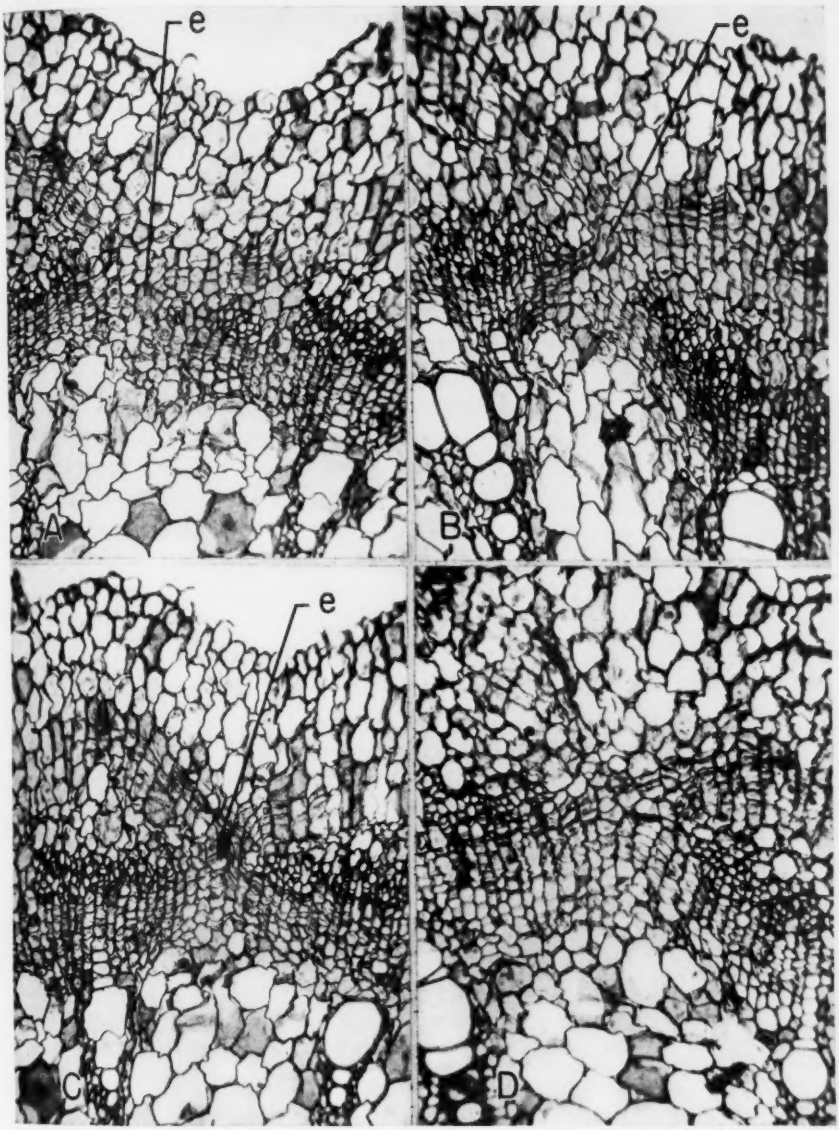

FIG. 15.—Details of development of connection of proliferating cells of endodermis with cambium of stele through activity and differentiation of ray cells. *A*, embryonic ray cell; *B*, first division of same; *C*, ray cells which have become meristematic on a line with proliferating endodermal cells over two adjacent bundles and just above similar cells in which interfascicular cambium is arising; *D*, entire group of embryonic cells forming continuous cambiums.

as to the actual substance or substances present within the cell which are responsible for the cell responses and tissue patterns which are differentiated in the case of indoleacetic acid, tryptophane, or various others, when supplied externally. There is evidence, however, that there are substances which—whether or not they show auxin activity when tested according to the *Avena* method (12)—do result in definite and characteristic responses in proliferation and subsequent patterns of differentiation when supplied externally to a rather wide variety of plants, either in a mixture with lanolin or in aqueous solution (4, 12, 16). This calls into question the value of the *Avena* test method as a precise or accurate means either for measuring or for determining quantitatively or qualitatively the substances responsible for the development of tumors caused by *Bacterium tumefaciens* (8, 9, 15), root nodules associated with rhizobia (7, 17, 18), or similar structures—provided auxin activity as thus measured is assumed to be primarily an indicator of the causes of the responses shown. It may well be that some of the substances involved may be measured in part, but it is just as likely that there are other effective compounds which escape measurement. Of the possible number of compounds which may be involved, some may show little or no auxin activity according to the *Avena* test. The complexities involved and already on record need no repetition here. Whether indoleacetic acid is or is not primarily responsible for the cellular responses observable in crown gall can no more be determined by such a test method (15) in itself than solely by the observance of the developmental sequences following treatment of plant parts with a 3 per cent lanolin-indoleacetic acid mixture or a 0.0001 per cent solution of it in water (16). The results following treatment with tryptophane as here presented indicate that it might well play a part. Its occurrence is general in living cells, and its concentration within any given cell or tissue might well fluctuate widely under various circumstances. The effect of growth-regulating substances within the plant, or outside it but in contact with it, may exert definite effects on the mobilization or accumulation of tryptophane, as has been indicated for more generalized groups of compounds (11, 12, 13, 16). The effects of varied conditions of environment, or planes of metabolism, and of the activity of organisms within or adjacent to the living tissues, are likewise important factors. To what extent tryptophane might become concentrated in tissues, and what would be the effects on development of such varied concentrations, are speculations which may well wait upon further experimental evidence, but consideration of the matter should not be omitted in relation to problems concerning growth and differentiation.

Critical data to show to what extent indoleacetic acid may be responsible for the development of the tubercles on the roots of leguminous plants in association with rhizobia are meager. Some of the suggestions are based upon the measure-

ment of auxin activity according to the *Avena* test, others on the occurrence of excresences on roots of leguminous plants following treatment with indoleacetic acid. Neither method furnishes either effective or sufficient evidence (**7, 8, 17, 18**). The course of development of tubercles on roots of leguminous plants has been partially described by several investigators (**10, 20, 21**). Through the courtesy of Dr. LOUISE WIPF I have had opportunity to observe a series of preparations which, while incomplete, indicate clearly—as do the results of the investigations above—that the tubercle or nodule is not a potential lateral root whose development has been arrested. In the slides examined the early cellular proliferations are in the cortex, and the subsequent differentiation of the vascular bundle—which is closely associated with the mass of cells containing the rhizobia and others which are actively proliferating—is very similar to the pattern followed in the differentiation of vascular bundles from the proliferated cells in the bean stem after treatment with the lanolin-tryptophane mixture. Especially striking is the similarity between the sequence of steps resulting in the ultimate connecting up of the bundles through the pericycle to the xylem and phloem of the stele of the root to the sequence followed in the stem when a comparable connection is made through the activity and differentiation of the cells of the ray (fig. 15). This is not to say that tryptophane may be the primary causative agent or substance in tubercle formation and differentiation, any more than indoleacetic acid may or may not be. It is possible that both are concerned, and other substances as well. But it is clear that the usual tests for auxin activity are far from adequate in arriving at a decision. So far as I am aware there is no recorded detailed account of histological studies on the reactions of the roots of leguminous plants to growth-regulating substances. A short time spent on the preparation, study, and interpretation of suitable material could and would definitely establish the course of development and the histological pattern of the nodule when rhizobia are present. Similar studies in relation to growth-regulating substances would furnish suggestions as to their possible direct relation to nodule formation or their effects upon the mobilization of those substances which are directly involved.

It is not essential to adduce other examples here, but it is evident that the explanations relating to the problems of tissue development and differentiation should not be oversimplified, nor based on results from the employment of any single technique. Generalizations must await additional detailed evidence from the chemical, physiological, developmental, and histological points of view. What significance these various results may eventually have in studies relating to cancer can be indicated better when many more of them are available.

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ANTHEROZOIDS OF SOME OF THE CHARACEAE

W. N. STEIL

(WITH SIX FIGURES)

Introduction

The antherozoids of the Characeae have been studied by a number of workers, including BELAJEFF (1), MOTTIER (3), MEVES (2), MÜHLDORF (4), and SASAKI (5), and there is considerable difference of opinion, not only in regard to the nature of the body but also in regard to the attachment of the cilia. It was the object of this investigation to obtain more knowledge of the antherozoids of some of the Characeae.

Material and methods

A number of species of *Chara*[1] and a single species of *Nitella*[1] were collected in the vicinity of Milwaukee, Wisconsin, between the middle of June and the middle of September, and brought into the laboratory for the study of both the living and the fixed and stained antherozoids.

Portions of the main axis of a plant bearing dwarf branches with the reddish colored antheridia were removed with small scissors and washed in tap water. A branch bearing the sex organs was next removed from the main axis and placed into a small amount of water on a slide. With the aid of dissecting needles, an antheridium was detached and transferred to a drop of water on another slide, and opened with the needles so that the antheridial filaments were exposed. This slide was placed into a petri dish, the inner surface of the cover of which had been moistened with water. The slide was removed at intervals during a period of about 40 minutes and examined under the low power of the microscope.

It was usually possible to determine the stage of maturity of the antherozoid, which when ripe is composed of distinct coils. When the antherozoids were observed to be actively motile, they were studied in the free swimming condition, or they were fixed and stained. The study of the actively motile antherozoids was facilitated by the fact that all the antheridia of the branches at a node usually reach maturity at the same time.

It was possible to make a more detailed study of the antherozoids in prepared slides. They were fixed in the usual way by inverting a slide with a drop of water containing motile antherozoids for about 2 minutes over the fumes of a 2 per cent osmic-acid solution. The slide was then set aside to dry.

[1] One of the species is undoubtedly *foetida;* the other species of *Chara* and the species of *Nitella* remain unidentified.

The technique for staining the antherozoids was similar to that already described (8). The schedule was as follows:

1. Place slide into a saturated tannic-acid solution in a Coplin for about 1 hour. This step may be omitted, but the solution facilitates staining the cilia.
2. Rinse in water.
3. Stain with safranin for 2–4 hours.
4. Rinse in water.
5. Dip slide into an acidulated alcoholic solution prepared by adding one drop of concentrated hydrochloric acid to 100 cc. of 95 per cent ethyl alcohol.
6. Rinse in water.
7. Stain with fast green.
8. Dehydrate at once with absolute ethyl alcohol.
9. Clear in clove oil.
10. Complete the slide in the usual way.

No observable alteration in the nature of the body or in the thickness of the cilia of the antherozoid was produced by the tannic acid. The safranin was obtained from the Coleman and Bell Co., certification number CS-6. One gm. of the dry stain was dissolved in 100 cc. of 50 per cent ethyl alcohol. The fast green was obtained from the National Analine and Chemical Co., certification number NGF-2. One-half gm. of the dry stain was dissolved in 100 cc. of 95 per cent ethyl alcohol.

The use of the acid solution is important since it not only destains the slime discharged from the antheridium and the cytoplasmic portions of the antherozoid, but it intensifies the stain in other parts of the body. A number of other combinations of stains were tried but none were found so satisfactory as the safranin-fast green combination. The nucleus, the "plastids" in the vesicle, and a small nearly spherical body at the extreme anterior end, all stain a brilliant red; other portions of the body and the cilia stain green.

Observations

The antherozoids were occasionally discharged immediately after the dwarf branch bearing antheridia was placed on the slide, but usually a few minutes elapsed before they emerged, the time interval depending upon their maturity. In most instances motile antherozoids were obtained within 20 minutes; they were never obtained later than 1 hour.

As is well known, a portion of the wall of each of the mother cells of the antheridial filament becomes gelatinized, forming a pore through which the antherozoid is set free. The change in the wall occurs when the antheridium has opened and the gelatinous substance within it and completely surrounding the filaments has

come into contact with the water. Although the origin of this substance has not been determined, it is probably the result of dissolution of the walls of the antherozoid mother cell. A study of the form and motions of the antherozoids is not difficult, on account of their large size and because the slime at first retards their movements.

An antherozoid, at first tightly coiled, assumes when set free a slow clockwise (figs. 1, 2) or counter-clockwise rotary motion (figs. 3–5). The number of coils varies from about three to four. The rotary motion becomes rapid only after it emerges from the slime. The rapidity of the movement soon decreases as the antherozoid becomes more loosely coiled (fig. 3), and ceases when it becomes completely uncoiled. The period of time of its free swimming motion is short, usually only 3–5 minutes. Since antherozoids which are discharged naturally also exhibit free swimming motions for similar short periods of time, the technique employed here evidently did not affect the duration of their swimming movements.

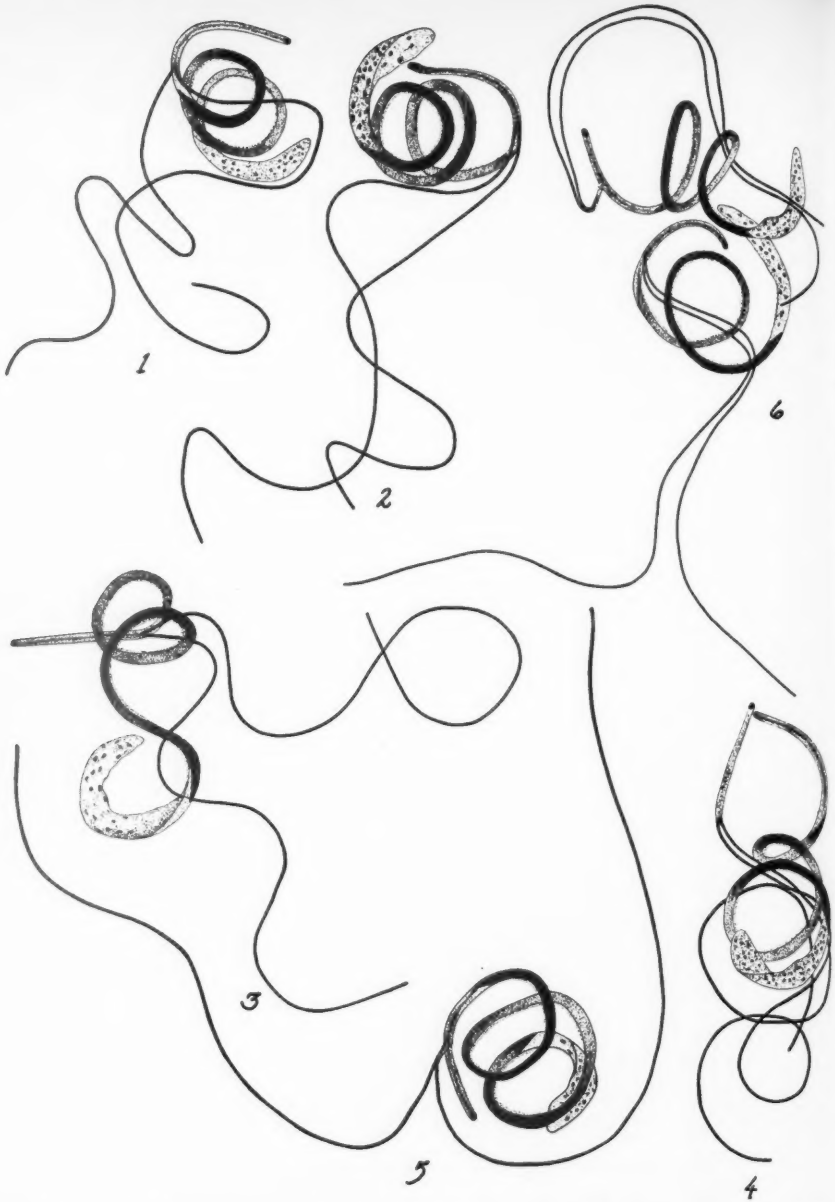
The movements of the cilia can readily be followed in the living antherozoid, although it is not possible to observe the differences in their lengths. That the cilia are attached for a considerable distance back of the anterior end, however, can be observed during the whole period of active motion of the antherozoid.

The body of the antherozoid is somewhat bandlike. The elongated nucleus (figs. 1, 2) extends from a point a short distance posterior to the place of attachment of the cilia to the large vesicle. The anterior end of the nucleus is considerably smaller in diameter than the posterior end. Both ends taper to a sharp point. A considerable portion of the anterior end of the antherozoid is cytoplasmic, as is shown in all of the figures.

The large vesicle (figs. 1–6) at the posterior end can readily be observed, even in the living antherozoid. After the antherozoid has been active for a few minutes it becomes somewhat spherical, then collapses, and is finally lost. The vesicle contains numerous red staining bodies which have been described by some workers as plastids. These "plastids" in the vesicles of different antherozoids vary in form, size, and number. In the vesicle of the antherozoid shown by figure 2 they are not only numerous but also large. To retain the vesicle it is necessary to fix the antherozoid a short time after it has been set free. A cytoplasmic portion (figs. 1–6) which extends the length of the antherozoid and completely surrounds the nucleus can be seen in the stained antherozoid.

The foregoing observations in regard to the nature of the body of the antherozoid, confirmed by the writer, have been made by a number of investigators.

The presence of a small, nearly spherical body (figs. 1–6) at the anterior end, not heretofore described in the Characeae, can be observed in the slides which have been prepared according to the technique described. Its origin and function have not been studied.



FIGS. 1–6.*—Figs. 1–5, *Chara:* Fig. 1, actively swimming antherozoid showing nucleus, cytoplasmic portion containing small spherical body, and lightly staining thread; the two broad cilia are of unequal length; the vesicle at posterior end containing numerous "plastids" and the plasmic portion on "inner" side of antherozoid are also shown. Fig. 2, antherozoid in free swimming condition similar to that of fig. 1; "plastids" in vesicle much larger than those in fig. 1; cilium at left is the longer. Fig. 3, outstretched antherozoid which has ceased its forward rotary motion. Fig. 4, antherozoid from which ciliary band has been removed; small body at anterior end shown in the band. Fig. 5, antherozoid showing ciliary band partially detached. Fig. 6, *Nitella:* ciliary band in one antherozoid partially removed. ×1750.

* Drawings made with aid of camera lucida.

The cilia, as has been described by most investigators, are attached at the same point a short distance anterior to the forward end of the nucleus. As already reported (9), they are of unequal length. They appear to be attached to a band—or are united to form a band—which extends to the anterior end of the body of the antherozoid and which is in close contact with this other cytoplasmic portion. The band, when detached, is about the same width as the anterior portion of the body. The cilia are unusually thick and together equal the band in width. Occasionally in the preparation of the slides the band was partially or completely detached from the anterior end of the body (figs. 4–6). When a needle was moved about in the drop of water containing motile antherozoids, often the band became partially or wholly detached from the body (fig. 4). The thick, elongated cilia probably adhered to the needle and hence the band was readily separated. The red staining body, already described, always remained in this detached band (fig. 4).

The cilia-bearing band, which has been the principal subject of this study, was described by MÜHLDORF (4) as an extension of the anterior end of the body. According to his description the antherozoid, at first closely coiled when set free from the mother cell, becomes outstretched, and the portion of the band in close contact with the anterior end unfolds and extends forward before the antherozoid begins its active swimming movements. According to MÜHLDORF, therefore, the cilia are attached at the anterior end and not some distance from it. SASAKI (5) is in agreement with MÜHLDORF. As the result of careful study, however, the writer could not confirm these observations.

A faintly staining thread (figs. 1–6) was frequently observed in the anterior end of the body. It could be more readily distinguished in those antherozoids which were slightly destained in the acid-alcohol solution. No definite statement can be made concerning the nature and origin of this structure and the small body at the anterior end until a study of spermatogenesis is made.

The antherozoids of the single species of *Nitella* (fig. 6) studied, although somewhat smaller, closely resemble those of the *Chara* species. The figure represents antherozoids having a clockwise rotary motion.

The antherozoid of the Characeae is similar to that of *Riccardia*, in which a difference in length of the two cilia was first reported by the writer (7), and to that of some other Jungermanniales reported by SHOWALTER (6) and MÜHLDORF (4).

Summary

1. The antherozoid of the Characeae studied is composed of about three to four coils. Its body is a somewhat flattened band which consists of an anterior cytoplasmic portion, a posterior vesicle, a cytoplasmic envelope, and an elongated nucleus which tapers to a sharp point at each end.

2. The anterior cytoplasmic portion consists of an outer and an inner bandlike part. The outer band bears the two cilia of unequal length and a small nearly spherical body at its forward end. In the inner portion a delicate thread can be seen.

3. The swimming motion of the antherozoids of *Chara* and *Nitella* is rotary, either clockwise or counter-clockwise.

4. The antherozoids of the different species of *Chara* studied are similar in every respect. The antherozoid of the *Nitella* species resembles that of *Chara*, but is considerably smaller.

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MECHANISM OF MITOSIS IN POLLEN TUBES

KARL SAX AND J. G. O'MARA

(WITH TEN FIGURES)

The mitotic division in the generative cell of the pollen tube of *Lilium* and many other genera has long been known to be unique. Although investigated frequently since the early work of STRASBURGER and NAVASHIN, certain features of this division remain obscure in spite of an abundance of relevant data. The nature of the spindle mechanism and the formation of an equatorial plate at metaphase are problems of considerable importance in relation to the mechanism of mitosis. In the division of the generative nucleus in pollen tubes of *Lilium*, a well-defined spindle has been described by GUIGNARD (3), STRASBURGER (12), HERRIG (4), and COOPER (1), but no spindle fibers were observed by SARGENT (9), NAVASHIN (6), WELSFORD (16), O'MARA (8), and WULFF (17). According to COOPER the chromosomes always form a normal equatorial plate, but irregular orientation of the chromosomes at metaphase has been described by STRASBURGER (12), NAVASHIN (6), WELSFORD (16), HERRIG (4), O'MARA (8), and WULFF (17). The results of TRANKOWSKY (14) are especially interesting, since they show that no type description will fit all nuclear divisions in the pollen tube. In *Galanthus* and *Convallaria* the division is irregular, with no evident spindle fibers and no regular equatorial plate. In *Hemerocallis flava*, however, an equatorial plate and a typical spindle are formed. Their occurrence in this species seems to be related to the size of the elements involved. In the other cases the tube is too narrow to accommodate a division figure of normal proportions, and an atypical figure is formed. According to UPCOTT (15) the division of the generative nucleus in *Tulipa* is regular, although the equatorial plate may be oblique or buckled.

Much of the disparity in the descriptions of mitoses in pollen tubes is due to differences in the materials used, although different results have been reported for the same species. Evidently variations do occur, and it is the irregular behavior that is of particular interest in relation to the mechanics of mitosis. In dealing with the features peculiar to this division, consideration must be given to the general process of which this particular division can be only a modification.

Divisions of the generative nucleus have been examined in the pollen tubes of *Lilium regale* and other species, *Tradescantia paludosa*, *Bellevalia romana*, *Scilla* sp., *Gasteria* sp., and *Zephyranthes* sp. *Lilium* pollen tubes were fixed in the styles at various times after pollination. Those fixed in alcohol-acetic were dissected out of the style and stained with aceto-carmine. Others were fixed with Flemming's solution and other standard fixatives, sectioned, and stained with Heidenhain's

haematoxylin. Some of the *Lilium* pollen grains were grown in stigmatic fluid, and at the time of nuclear division the tubes were fixed in aceto-carmine. Pollen of the other genera were grown on slides, following NEWCOMER's (7) technique, and were fixed and stained with aceto-carmine or fixed with a modified Flemming's solution and stained with the triple stain or with Heidenhain's haematoxylin.

The analysis of the generative nucleus division in *Lilium* was done by the junior writer while he was a graduate student at Harvard University.

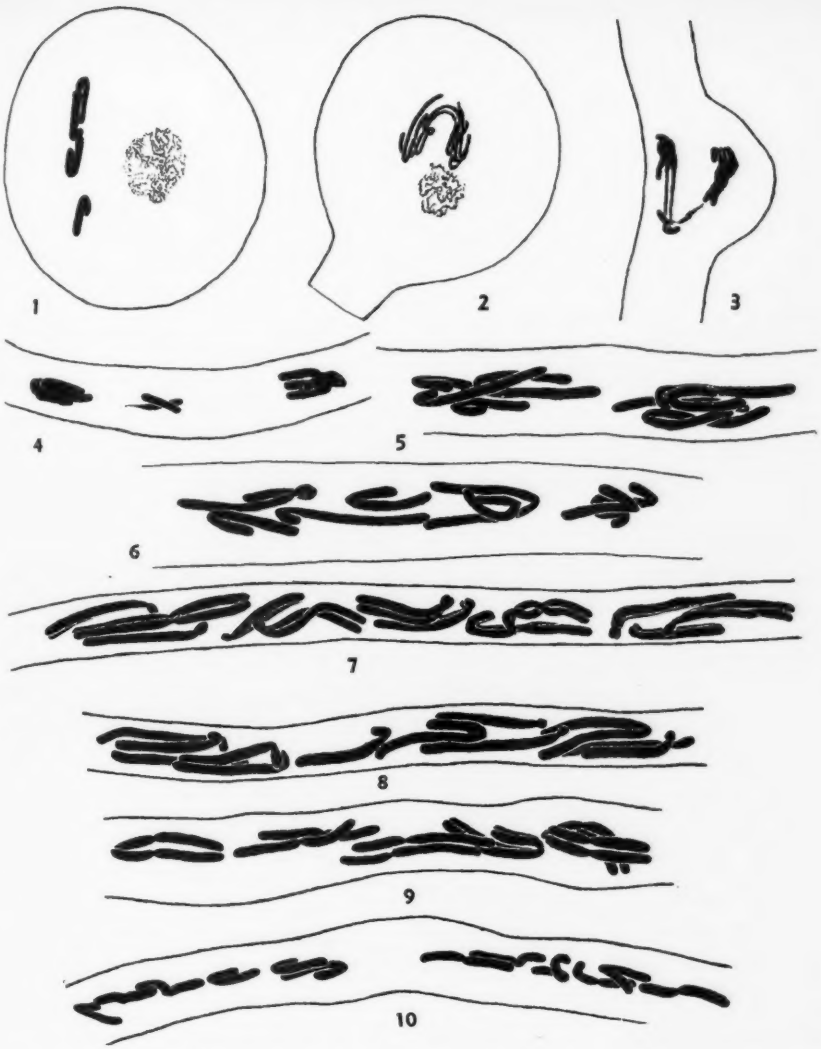
DIVISION OF GENERATIVE NUCLEUS

Of the various genera examined, normal division figures were characteristic in *Bellevalia*, *Scilla*, and *Zephyranthes*. The centromeres were oriented on a rather definite equatorial plate at metaphase, with the chromosome arms extending lengthwise in the pollen tube. At anaphase the daughter chromosomes were uniformly distributed to the poles in a regular manner. No clearly defined spindle fibers were observed, but a definite spindle mechanism is operative.

A number of exceptional division figures were observed in *Bellevalia*. The generative nucleus is compact and stains intensely in the mature pollen grain, and the tube nucleus appears to be disintegrating. Occasionally the individual chromosomes are clearly observed, indicating their orientation in the compact generative nucleus (fig. 1). Since the generative nucleus resembles the gametic nuclei in structure and staining capacity, it seems probable that in both cases the chromosomes are more contracted and rigid than they are in a typical resting nucleus. When *Bellevalia* pollen grains are cultured on agar for several hours, the generative nucleus often divides in the pollen grain. As a rule these divisions are abnormal in two respects: the spindle is curved so that the anaphase chromosomes form a crescent or U-shaped figure, and the chromosomes do not reach their normal degree of contraction (fig. 2). Often the chromosomes are so long that complete separation at anaphase is impossible. Apparently the spindle formation is precocious, so that the nuclear division is initiated before the minor coiling mechanism has effected the normal degree of chromosome shortening characteristic of a normal metaphase.

The bent spindle, as indicated by the behavior of anaphase chromosomes, is also found in occasional abnormal bulges in the pollen tube of *Bellevalia* (fig. 3). Evidently the spindle is always curved when space permits, either in the pollen grain or in swollen regions of the pollen tube. In normal pollen tubes the spindle is held within the limits of the narrow tube, and little or no bending of the spindle is possible. These spindles are very long, as indicated by the distance separating the daughter chromosomes at anaphase (fig. 4).

In most mitoses the centromeres of the anaphase chromosomes are oriented toward their respective poles, but in these pollen-tube divisions one or both chromosome arms often pass to the poles in advance of the centric region of the chromo-



FIGS. 1–10.—Camera lucida drawings of generative nuclei divisions from aceto-carmine preparations. Figs. 1–6, *Bellevalia:* 1, chromosomes of generative nucleus in pollen grain; 2, precocious division of generative nucleus in pollen grain showing elongated chromosomes and effect of bent spindle; 3, bent spindle in bulge of pollen tube; 4, anaphase in pollen tube showing wide separation of daughter nuclei and failure of acentric fragments to pass to poles; 5, same showing orientation of free chromosome arms toward one pole; 6, exceptional type of chromosome separation at anaphase. Fig. 7, metaphase in pollen tube of *L. regale*. Fig. 8, one pole at anaphase in pollen tube of *L. regale*. Fig. 9, chromosomes separating at metaphase in pollen tube of *T. paludosa*. Fig. 10, anaphase in pollen tube of *T. paludosa*.

some (figs. 5, 6). In rare cases most or all of the chromosomes at one pole are oriented in the same way as the sister chromosomes at the opposite pole (fig. 5). As a rule the centromeres of an anaphase group of chromosomes are at about the same plane in the cross section of the pollen tube, but occasionally they are scattered along the spindle as in *Lilium* and *Tradescantia* (fig. 6).

Both normal and abnormal division figures were found in pollen tubes of *Lilium regale*, regardless of the technique or the fixatives used. When the tubes were wide the chromosomes tended to become oriented on an equatorial plate at metaphase and to pass to the poles in unison. But in narrow tubes the chromosomes were distributed along the pollen tube at metaphase and were not grouped at anaphase (figs. 7, 8). Such figures were found both in cultured pollen tubes and in those growing in the style, although the cultured tubes were wider and more of the division figures were normal. It is obvious that the chromosomes shown in figure 7 could not possibly form an equatorial plate within the limits of the pollen tube. Nor can this stage be referred to as anaphase, as several critics have done in referring to O'MARA'S earlier photographs. Although relational coiling of sister chromatids is found in one chromosome, the chromosomes have reached their normal degree of contraction, as indicated by a comparison of the anaphase figure shown here. Such irregular metaphase figures are common in pollen tubes of *L. regale*, *L. superbum*, and *L. canadense*. Only normal figures were found in cultured pollen tubes of *L. speciosum rubrum*.

No well defined spindle mechanism could be observed in any of our preparations of *Lilium*, although no attempt was made to differentiate such structures with special fixing and staining techniques. A spindle mechanism associated with the centromeres was demonstrated indirectly. Pollen grains were X-rayed and acentric chromosome fragments were produced. At anaphase in the pollen tube such acentric fragments did not pass to the poles but remained in the cytoplasm between the daughter nuclei. Spontaneous acentric fragments in *Bellevalia* behave in the same way (fig. 4). The centromere is essential for nuclear division, even in cases where the chromosomes are scattered at metaphase, and where the spindle is not typical. Failure to demonstrate a well-defined spindle presumably is due to unusual cytoplasmic conditions, but under certain conditions of fixation it may be evident (1). According to COOPER, nine of the twelve *Lilium* chromosomes have subterminal fiber constrictions and three have more or less median centromeres. We find ten chromosomes with subterminal centromeres and only two with submedian constrictions (figs. 7, 8).

Pollen tubes of *Tradescantia* grown on agar medium with 8 per cent sugar provide many abnormal division figures of the generative nucleus. Often the chromosomes are distributed along the tube and divide without even approaching an equatorial plate stage (fig. 9). In fact it is difficult to find a typical metaphase plate stage in most of these preparations. Although the chromosomes are not so

well separated as they are following acenapthene treatment, as described by SWANSON (13), they often are far enough apart so that little overlapping occurs.

These widely distributed chromosomes divide and the daughter chromosomes pass to opposite poles. Such division necessitates the passage of chromosomes in opposite directions at various levels along the spindle. At later anaphase stages the chromosomes are still separated and do not pass to the poles together (fig. 10). Although division and separation of the chromosomes are synchronized, each chromosome appears to behave as an independent unit. Frequently the distal ends of the chromosome arms precede the centric regions toward the poles at anaphase (fig. 10), as is found in both *Lilium* and *Bellevalia*. This behavior does not mean that the centromere is inactive at anaphase. The chromosome arms may be unable to turn in the narrow tube, or cytoplasmic streaming may at times carry the distal end of the chromosome to the pole in advance of the centromere. Observations on living material show that the cytoplasm streams rapidly in the pollen tube, although it was not possible to determine the condition of the cytoplasm in the region of the spindle.

The abnormal divisions of the generative nucleus in *Lilium* and *Tradescantia* are related to the nuclear configuration and the spatial limitations in the pollen tube. The generative nucleus in the pollen grain is crescent- or spindle-shaped in longitudinal section. The elongated nucleus passes down the pollen tube, and in late prophase the chromosomes are spaced along the nucleus so that their centromeres may be separated by the length of several chromosomes. Congestion on a metaphase plate is prevented by spatial limitations. When space permits, the chromosomes are able to congregate on an equatorial plate even though rather widely separated at prophase.

The elongated generative nucleus in *Tradescantia* seems to be subject to the stress of cytoplasmic streaming, and it may be divided while in early prophase. In such cases the chromosomes in the two parts of the nucleus may at times develop at different rates, so that one group is at metaphase and the other still in prophase.

When the chromosomes are too widely scattered in the pollen tubes of *Tradescantia* they are unable to divide. They contract and become pycnotic, ultimately disintegrating in the cytoplasm. Apparently a certain degree of association is necessary for the control of division processes and the functioning of the spindle.

MECHANICS OF NUCLEAR DIVISION IN POLLEN TUBE

The division of the generative nucleus in the pollen tube may be atypical in several respects. The spindle often is very long and if space permits it may be bent, the chromosomes can divide without the formation of an equatorial plate, anaphase chromosomes can pass to the pole even when widely scattered along the tube, and the distal ends of the chromosome arms may precede the centric region in passing to the pole. Contrary to some of the earlier conclusions, a spindle mech-

anism is operative, although it is not so clearly differentiated as it is in typical somatic divisions. The centromere controls the division and separation of the chromosomes in the division of the generative nucleus as it does in other mitoses.

The long spindle is characteristic of nuclear divisions in the pollen tubes of many species. UPCOTT (**15**) has suggested that the long spindle is due to the difficulty of chromosome orientation on an equatorial plate. Proper orientation is considered necessary for spindle functioning, and if orientation is delayed the spindle grows until the chromosomes can separate. Apparently spindle formation may not be completely synchronized with chromosome development and division. In *Bellevalia* the chromosomes may be completely contracted and fail to divide because no spindle mechanism is available (fig. 1), or the chromosomes may be separated by a precocious spindle before the chromosomes are contracted sufficiently to permit complete separation at anaphase (fig. 2). It is known that chromosome division, nuclear division, and cell division are independent processes although normally they are harmoniously synchronized (**10**). The various processes involved in nuclear division also are synchronized in normal mitosis, but those of chromosome development are not always synchronized with spindle formation in the divisions of the generative nucleus, and to a certain extent the two processes are independent. The synchronization of nuclear and cytoplasmic processes is a normal feature of mitosis, but the timing of chromosome development and spindle formation regularly differs in meiosis. This regulated change is responsible for the difference between mitosis and meiosis (**2**). The synchronization of cellular activities is in accord with OSTERHOUT'S suggestion that life is dependent upon a series of processes whose rates bear a definite relation to one another.

The second atypical variation in the division of the nucleus in the pollen tube is the division and separation of the chromosomes without the formation of an equatorial plate. Such behavior is difficult to reconcile with the theory of a repulsion equilibrium which forces the centromeres to lie midway between the poles in an equatorial plate (**2**); nor can the scattered chromosomes, as shown in figures 7 and 9, be considered as lying on an oblique or buckled plate, as UPCOTT (**15**) has suggested for less extreme cases in *Tulipa*. It is possible that the great elongation of the spindle, characteristic of pollen-tube divisions, is sufficient to include all the scattered chromosomes and subject them to the spindle influence. Any theory of changes involving centromeres and centrosomes has to account for the behavior of daughter chromosomes at anaphase. A comparison of figures 9 and 10, for example, shows that chromosomes must pass each other on their way to opposite poles. Such behavior cannot be reconciled with a change of equilibrium at the spindle poles, nor can it be attributed to changes in the shape of the spindle due to a repulsion of its polar regions. In these aberrent divisions of the generative nuclei chromosome behavior is synchronized, but each chromosome appears to be capable of

independent division and separation, although all may be under the influence of a common spindle mechanism. In certain respects the apparent independence of the chromosomes in anaphase separation is similar to the independent chromosome spindles in certain coccids, as described by SCHRADER (11).

When the chromosomes become too widely separated in the pollen tube they are unable to divide, suggesting that they must be included within the range of spindle activity to continue normal development.

The third abnormality of the division in the pollen tube is the frequent failure of the centromere to precede the chromosome arms to the poles. The passage of the daughter chromosomes to the poles with the distal ends in advance might appear to be caused by cytoplasmic streaming, but the cytoplasm of the generative cell appears to be homogeneous and unlike the actively streaming cytoplasm of the pollen tube. It is more probable that, if chromosome arms cannot turn in the narrow tube, the force at the centromere simply pushes them toward the pole. These cases, however, are similar to the behavior of anaphase chromosomes in *Sciara* (5), where spatial limitations do not appear to be involved.

It is evident that no type description will fit all nuclear divisions in pollen tubes. In certain genera practically all the divisions may be normal, as we have found in cultured pollen tubes of *Bellevalia*, *Scilla*, and *Zephyranthes;* while other genera, such as *Lilium* and *Tradescantia*, have a high frequency of atypical mitoses. Within a genus some species may have regular divisions and others irregular, as is true in *Lilium*. Even in the same cultures of a given species some pollen-tube mitoses are normal and others atypical. No doubt environmental factors are of importance in determining the type of nuclear divisions, since COOPER (1) found only regular divisions and we find many irregularities in divisions of the generative nucleus of *L. regale*. The effect of environment is most striking in a comparison of pollen tubes of *Narthecium* grown in culture and grown in the styles (18). The cultured tubes are much wider and the division is normal, while those in the style are narrow and no equatorial plate is formed at metaphase. When space permits, the chromosomes pass on an equatorial plate at metaphase and divide regularly at anaphase. When the pollen tube is too narrow to permit free orientation of the chromosomes, they divide without passing on to an equatorial plate and pass irregularly to the poles at anaphase.

Summary

The type of division found in pollen tubes varies with different genera, species, and among pollen tubes of the same culture. Division of the generative nucleus is generally normal in *Scilla*, *Bellevalia*, and *Zephyranthes*, while atypical divisions are frequent in pollen tubes of *Tradescantia*, *Gasteria*, and *Lilium*. When spatial relations are adequate the chromosomes form on an equatorial plate and the ana-

phase chromosomes pass to their respective poles in unison; but large chromosomes in a narrow pollen tube divide without formation of an equatorial plate, and the daughter chromosomes appear to act as independent units as they pass irregularly to the poles. These divisions are dependent upon the action of the centromeres and a spindle mechanism. Very long spindles are often found in divisions of the generative nucleus, and in *Bellevalia* they are curved or U-shaped when the division occurs in the pollen grain or in bulges in the pollen tube. The relation of these abnormal divisions is discussed in relation to the mechanics of mitosis.

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CURRENT LITERATURE

Geology of Coal. By OTTO STUTZER. Translated and revised by ADOLPH C. NOÉ. Chicago: University of Chicago Press, 1940. Pp. xiii+461. Figs. 198. $5.00.

Those interested in the highly varied scientific aspects of coal will find this book extremely useful as a reference. The study of coal has been subject to more than its share of provincialism, and this translation and revision will be of particular value in making facts on coal from European sources generally available.

The subject matter appears to be treated adequately and fairly, although in a few instances opinion may vary. The characterization of Carboniferous plants listed for ecological comparison (p. 169) may include some exceptionable items; the statement that there were no climatic zones in the Carboniferous (p. 172) would seem to need qualification. Paleobotanists will be especially interested in the chapters on coal microscopy, origin of coal beds, and parts of the chapter on stratigraphy of coal deposits. The nature and origin of coal balls is included in the chapter on petrography of coal beds.

Numerous references to the literature are given at the close of each chapter and are a great improvement over the footnote references, often incomplete, given in the earlier German edition. There are few important omissions. The paper by BLACKBURN and TEMPERLEY on the boghead algae (Trans. Roy. Soc. Edinburgh **58**:841–868, 1936) perhaps deserved mention, since it may be regarded as finally closing the long controversy as to the nature of the boghead algal bodies. Likewise the collection of papers resulting from the two Heerlen Congresses for the Advancement of Carboniferous Stratigraphy might well have been referred to specifically. The older literature seems best represented, but enough references to later work have been included to lead to authoritative works on nearly every topic.

The volume is concluded with an extensive general index, an author index, an index of places, and a list of European and American equivalents of weights, measures, and energy units (the decimal system is used throughout the text). These are of value and to a large extent offset objection of the organization of the book under too few chapter headings. For example, useful minerals associated with coal are treated in the chapter on stratigraphy, although this discussion has no direct bearing on the chapter subject. The complete indices obviate any difficulty in referring to this material, however; in fact, they give assurance of the permanent reference value of the volume.

Personal credit for the excellence of this book is probably not due to any one man. The primary collection of information was accomplished admirably by PROFESSOR STUTZER, but much additional information was incorporated in it by PROFESSOR NOÉ. The death of STUTZER in 1936 and of NOÉ in 1939—while actively engaged in the last of the work of translation—meant that further advice of a technical nature was needed for its successful publication. This service and the tedious work of checking the entire manuscript as a whole, indexing, proofreading, etc., was largely performed by DR. GILBERT H. CADY of the Illinois Geological Survey. His assistance was of inestimable value in completing this task.

The format is excellent, the numerous illustrations generally clear, and the book free from typographical errors.—J. M. SCHOPF.

The Course of Evolution. By J. C. WILLIS. Cambridge: University Press; New York: MacMillan Co., 1940. Pp. viii+207. $3.00.

This volume is a sequence of the author's *Age and Area*, which appeared in 1922 and which questioned the adequacy of natural selection of chance variations as a major factor in evolution. "Evolution and natural selection are probably to a great extent independent, and they work at right angles to one another, with (in plants at any rate) little mutual interference." "Definite single mutations which cause structural alterations, which may, but by no means must, have some functional advantage attached" bring about evolution. The course of evolution is in the downward direction, from family to variety, rather than upward, as required by the theory of natural selection. Evolution moves in the opposite direction to that required by natural selection and is therefore not a matter of chance, but of law which is not as yet understood. Chromosome alterations are thought to be largely responsible for mutations.

The questions of mutation, adaptation, isolation, differentiation, and divergence of variation as factors in evolution are discussed at some length and 34 text cases presented, based upon number of species, morphological and taxonomic characters, and geographical distribution in support of his thesis. The book makes interesting and stimulating reading and is thought provoking, whether or not one agrees with the author.—J. M. BEAL.

Manual of Cultivated Trees and Shrubs Hardy in North America Exclusive of the Subtropical and Warmer Temperate Regions. By ALFRED REHDER. New York: MacMillan Co., 1940. Pp. xxx+996. $10.50.

This second edition of REHDER's excellent treatise needs no recommendation to a public which has used the first one enthusiastically. Introduction of new species and varieties, hybridization, revisions in certain groups as a result of systematic research at the Arnold Arboretum and elsewhere, nomenclatural changes made necessary by the revision of the International Rules in 1930 and 1935, as well as the exhaustion of the first edition, make apparent the need for the present book.

This volume embodies the same general features as the earlier edition, but is approximately 70 pages longer and now includes 2535 fully described and keyed species or hybrids, with about 2685 varieties or lower taxonomic categories, distributed among 486 genera and 113 families. These numbers are in contrast to the 468 genera, 2350 species, and 2465 varieties fully treated in the first edition. In addition, brief treatments of about 25 genera, 1400 species, and 540 hybrids are appended to the units with which they are most closely related. The climatic zone map, based on average annual minimum temperatures, has been revised thoroughly, and now appears enlarged and in two colors as a frontispiece. New data on hardiness assembled in the last 12 years have been incorporated in the species descriptions in accord with the zones now recognized. The list of authors' names has been enlarged; also the glossary, and list of references to illustrations. Family arrangement still follows the ENGLER and PRANTL sequence, with few exceptions, the most important of which is the transfer of the monocotyledons to the end of the book. In some cases generic and specific arrangement has been revised greatly. Double citations of authors' names have been adopted where necessary, in accord with present International Rules. The author has also abandoned his earlier practice of elevating each subdivision of a species to varietal rank, thus avoiding new combinations. In this edition, simple trinomials are used for species subdivisions of any category; and the rank assigned by the cited author, who first placed the subdivisional epithet under the accepted binomial, follows that author's name, even when REHDER does not agree with this rank. He announces his intention to issue a "biblio-

graphical supplement supplying for all the names mentioned in the Manual exact citations of their source and for trinomials the category considered to be botanically correct."—C. E. OLMSTED.

The Biochemistry of Symbiotic Nitrogen Fixation. By PERCY W. WILSON. Madison: University of Wisconsin Press, 1940. Pp. xiv+302. Illustrated. $3.50.

This interesting monograph deals with many of the problems of nitrogen fixation and the difficulties of permanent agriculture due to the gradual transfer of nitrogen from land to sea.

The introductory chapters present the long-range aspects of the nitrogen economy of man and of nature, including the history of legumes in agriculture from Grecian and Roman times to the present. The next chapters take up the chemistry of bacteria; the interaction of host and bacteria; the fixation of nitrogen by bacteria and plant; the carbohydrate-nitrogen relationships in symbiotic nitrogen fixation; the excretion of nitrogenous compounds by legumes; the chemical mechanism of the fixation process; and the physical-chemical characteristics of the enzyme system concerned in fixation. The final chapters consider practical applications of our knowledge of fixation, with some brief concluding remarks on the future of research in the field.

The book is well written, and is worthy of careful reading by anyone who wishes a comprehensive summary of the entire field of nitrogen fixation by bacteria and legumes. The 32 pages of literature citations will be useful in obtaining more complete treatment of particular phases. There are 34 full-page plates and 27 text figures. A short subject index adds to the usefulness of this valuable treatise.—C. A. SHULL.

If They Could Speak. 120 Broadway, New York: Chilean Nitrate Educational Bureau, 1941. Pp. 54.

This is an attractive handbook of the mineral deficiency diseases of plants. The entire story is told in colored reproductions made from Kodachrome transparencies—95 of them—taken in the field, with brief descriptive identifications accompanying the plates. The deficiencies included are of boron, calcium, copper, iron, magnesium, manganese, nitrogen, potash, and zinc. About 35 species of plants are portrayed, including many important crop plants, such as alfalfa, corn, tobacco, soybean, wheat, etc.

The Chilean Nitrate Educational Bureau has a fine conception of its opportunities for service, and this handbook is additional evidence of enlightened policy. Copies may be obtained merely by requesting them. The director of the Bureau, MR. HERBERT C. BREWER, would welcome comments on the reproductions from anyone who has first-hand knowledge of deficiency diseases of plants.—C. A. SHULL.

The Ferns and Fern Allies of Wisconsin. By R. M. TRYON, JR., N. C. FASSETT, D. W. DUNLOP, and M. E. DIEMER. Madison, Wisconsin: Department of Botany, University of Wisconsin, 1940. Pp. v+158. Figs. 215; 76 distribution maps. $1.00.

Combining excellent photographs of natural habitats and of herbarium plants, low magnification photomicrographs, drawings of leaves and spore-bearing structures, and distribution maps for nearly every species and variety growing in Wisconsin, this manual has achieved a new standard of excellence. The nomenclature follows the latest International Rules, with the often varying synonyms of GRAY'S Manual and BRITTON and BROWN'S Flora parenthetically included. The key to the genera and the key to species of ferns based on sterile leaves (which is well illustrated with labeled drawings and photographs) will satisfy the professional botanist and the

interested amateur. The description of each plant is short and critical. The distribution for Wisconsin and for other states and countries is given.—P. D. VOTH.

Moss Flora of North America North of Mexico. Vol. II, Part 4. By A. J. GROUT. Newfane, Vermont: Published by the author, 1940. Pp. 211+285. Illustrated. $2.50.

DR. A. LEROY ANDREWS treats the genera *Bryum* and *Rhodobryum*, the Mniaceae, and the Rhizogoniaceae in this concluding part of the flora. The successful treatment of the difficult genus *Bryum*, together with additional notes on the Bryaceae, an artificial key to *Pohlia* in the form of an appendix (by GROUT), as well as additions and corrections to all three volumes, make this last part especially valuable. Many of the plates were drawn or supplemented by SEVILLE FLOWERS.

Requiring 12 years for its completion (see previous reviews in BOT. GAZ. **88**:111, 1929; **93**:110, 1932; **96**:578, 1935; **101**:509, 1939), and having the cooperation of America's most successful amateur and professional bryologists, this flora supplies a great need in this hemisphere. State bryological floras will now have a basis for greater unity; professional bryologists will be saved endless effort; and, it is hoped, amateurs will be encouraged to begin the study of plants in general and of the mosses in particular.—P. D. VOTH.

Mechanisms of Biological Oxidations. By DAVID E. GREEN. New York: Cambridge University Press, 1940. Pp. 181. $2.75.

The oxidizing enzymes have probably been more intensively investigated in recent years than any other group. This book gives a detailed account of the properties, chemical nature, factors affecting and mode of action of those oxidizing enzymes whose prosthetic group contains respectively copper, iron porphyrin, zinc, flavin, nicotinic amide, thiamine, and glutathione. Certain enzymes whose prosthetic group has not yet been isolated are also treated. Wherever known, the steps in the oxidation of a compound are given, with the exact mode of action of the enzyme concerned. As the book shows, considerable progress has been made in putting the oxidation processes of the living organism on a purely chemical basis. Also the studies show some relationships between enzymes on the one hand and vitamines and trace metals on the other.

Biologists will welcome this critical summary of the literature of the oxidizing enzymes.—S. V. EATON.